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Psychological States as Factors in the Development of Malignant Disease: A Critical Review[1,2]

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Summary

The literature concerning the effect of personality structure on the etiology and development of neoplasms was reviewed. In the eighteenth and nineteenth century, publications repeatedly indicated the belief of their writers that emotional states had an important effect on the development of cancer. In the past 10 years a large number of studies in this area has been done with the modern techniques of psychiatry and clinical psychology. By and large, recent studies have come to the same conclusions as the eighteenth- and nineteenth-century oncologist. At present the data seem to justify 4 conclusions: 1) There seems to be a correlation between neoplastic disease and certain types of psychological situations. 2) The most consistently reported, relevant psychological factor has been the loss of a major emotional relationship prior to the first-noted symptoms of the neoplasm. 3) There appears to be some relationship between personality organization and the length of time between the appearance of a neoplasm and the death of the patient. 4) There may be some relationship between personality organization and the type or location of a cancer.—**J. Nat. Cancer Inst. 22: 1–18, 1959.**

The psychosomatic concept is a very old one, which has gained recognition and lost and gained again throughout its history. In the medieval era, the theory of body humors was a complex and sophisticated theory of mind-body interaction. A letter written in 1402 by a physician, Maestro Lorenzo Sassoli, to a patient had a curiously modern sound. ". . . let me speak to you regarding the things of which you must most beware. To get angry and shout at times pleases me, for this will keep up your natural heat; but what displeases me is your being grieved and taking all matters to heart. For it is this, as the whole of physic teaches, which destroys our body more than any other cause" (*55*). Cancer was frequently associated with the humoral theory. Galen (*see 28*) believed that melancholic women were more prone to cancer than those of sanguine temperament. Walshe, however, in reviewing the literature notes that

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"women of high color and sanguinous temperament are more subject to mammary cancer than are those of different constitution" (*72*).

Slowly emerging from the humoral theory, the eighteenth- and nineteenth-century physician had a training and an orientation that made him alert to apparent relationships between personality and neoplastic disease. As early as 1759, Gendron (*19*) stressed the importance in cancer of ". . . Disasters in Life, as occasion much trouble and grief." Kowal (*35*) has quoted from two of the cases which Gendron used to illustrate this point.

> Case I: Mrs. Emerson, upon the Death of her Daughter, underwent Great Affliction, and perceived her Breast to swell, which soon after grew Painful; at last broke out in a most inveterate Cancer, which consumed a great Part of it in a short Time. She had always enjoyed a perfect state of Health.
>
> Case II: The Wife of Mate of a Ship (who was taken some Time ago by the French and put in Prison) was thereby so much affected that her Breast began to swell, and soon after broke out in a desperate Cancer which had proceeded so far that I could not undertake her case. She never before had any Complaint in her Breast.

This concept that severe emotional trauma (to use modern terminology) contributed markedly to the onset and development of cancer was not regarded as radical. It was widely accepted and referred to by many writers as a well-known fact. It was, however, a fact which at the time had little clinical significance. There was no way that it could be used to help the patient. Clinical psychiatry did not begin to appear until the early twentieth century. Before that, anyone interested in mental phenomena could only observe, record, and classify. He had no tools with which to explore or intervene. Burrows (*4*), in 1783, noted that cancer is caused by: "The uneasy passions of the mind with which the patient is strongly affected for a long time, that weaken the circulation of the blood and consequently thicken it." In 1802, a group of the leading physicians in England and Wales formed an organization with the optimistic and cheerful name of "The Society for the Prevention and Cure of Cancer." They published a list of eleven questions, each describing an area in which they felt the need for further research. One of the questions was, "Is there a predisposing temperament?" (*26*).

Nunn, in 1822, published his widely used *Cancer of the Breast* (*53*) in which he made quite clear his belief that emotional factors can influence the growth of neoplasms. He describes a woman (Case 44) with "tumors of the right breast and nodules on the body." He continues: "18th January 1813. A general shrinkage of the tumors to a most remarkable degree. . . . This coincides with a shock to her nervous system caused by the death of her husband. Shortly thereafter the tumors again increased in size and the patient died."

In 1846, Walter Hoyle Walshe published his treatise *The Nature and Treatment of Cancer* (*72*). This became the definitive work of the period. He covered all that was then known about the subject. Walshe was a highly trained and respected man and ". . . apparently was conversant

with all that had been written and said about cancer to his time" (*35*). Walshe posed his viewpoint clearly and forcefully:

> Much has been written on the influence of mental misery, sudden reverses of fortune, and habitual gloominess of temper on the deposition of carcinomatous matter. If systematic writers can be credited, these constitute the most powerful cause of the disease; and Lobstein, assuming the fact as established, exercised his ingenuity in tracing the connextion of cause and effect: moral emotions produce defective innervation, this perversion of nutrition which in turn caused the formation of carcinoma. Whether this be the real catenation of circumstances or not, and although the alleged influence of mental disquietude has never been made a matter of demonstration, it would be vain to deny that facts of a very convincing character in respect to the agency of the mind in the production of this disease are frequently observed. I have myself met with cases in which the connextion appeared so clear that I decided questioning its reality would have seemed a struggle against reason.

Walshe made certain recommendations to members of families with a history of cancer, which illustrate the strength of his belief in this viewpoint. He advised them to use great care in their choice of professions, avoiding those ". . . the active and serious exercise of which entails a more or less constant care and anxiety. The importance of this consideration appears from what I have said on the influence of mental suffering in generating the disease. For this reason, the professions of the Bar, Medicine and Diplomacy should be avoided. . . .All things considered, the professions of the Army, Navy and the Church, unless there be some special objection, offer the best chance of escape from the diseases to individuals predisposed to cancer." By implication, Walshe was clearly stating that a genetic readiness plus a long-term psychological stress results in cancer.

In France, Chomel,[3] Bouveret, and others strongly stated what to them was an obvious connection. Amussat wrote: "The influence of grief appears to me to be, in a general way, the most common cause of cancer" (*1*).

In this country, Willard Parker summed up his 53 years of surgical experience with cancer (*57*):

> It is a fact that grief is especially associated with the disease. If cancer patients were as a rule cheerful before the malignant development made its appearance, the physiological theory, no matter how logical, must fall: but it is otherwise. The fact substantiates what reason points out.

Ephraim Cutter (*7*), at about this time, noted: "Mental depression is too often an element in cancerous cases to be overlooked." Cutter went much further. He believed that the disease could be cured by diet and by stimulating and exercising the patient's will to live. During the same year in which Cutter wrote, C. H. Hughes introduced the term "neurotic" into this field (*29*). Here was a neurologist speaking of the "neurotic origin and nature of cancer."

[3] "Les Chagrins prolonges paraissent avoir une grande influence dans le development du cancer." (Prolonged sadnesses appear to have a marked effect on the development of cancer.) Quoted by Foque (*17*).

Long before this, Sir James Paget, in his classic, *Surgical Pathology*, had written:

> The cases are so frequent in which deep anxiety, deferred hope and disappointment are quickly followed by the growth and increase of cancer, that we can hardly doubt that mental depression is a weighty additive to the other influences favoring the development of the cancerous constitution (*56*).

Sir Thomas Watson in 1871, phrased his conclusion as follows:

> Great mental stress has been assigned as influential in hastening the development of cancerous disease in persons already predisposed. In my long life of experience, I have so often noticed this sequence that I cannot but think the imputation is true (*73*).

Herbert Snow, working at the London Cancer Hospital, was deeply impressed by Paget's view as well as the reports of other predecessors. In three books written in 1883, 1890, and 1893, he presented in detail his research findings and his concepts (*64–66*). In his last book, he wrote:

> We are logically impelled to enquire if the great majority of cases may not own a neurotic origin? . . .We find that the number of instances in which malignant disease of the breast and uterus follows immediately antecedent emotion of a depressing character is too large to be set down to chance, or to that general liability to the buffets of ill fortune which the cancer patients, in their passage through life, share with most other people not so afflicted (*66*).

The first statistical study in this field was done by Snow. He reported on 250 successive outpatients and inpatients. In 156 of them, . . . "there had been immediately antecedent trouble, often in very poignant form, as the loss of a near relative . . . 32 spoke of hard work and deprivation . . . 43 had histories permitting a suspicion of mechanical injury . . . in 19 no common history was found." Snow presented his method and his cases in detail. Without question, his study was the finest that could be done with the psychological tools available at the time.

With Snow, we come to the end of an era: no longer was this psychosomatic concept commonly referred to in texts and treatises on cancer. A few reports continue,[4] but the idea of a psychic factor in the etiology of malignant disease pretty much vanished from the literature. The reasons for this are complex. It was not useful knowledge, since there were no tools or concepts which could be used to help the patient. Further, the psychosomatic concept had been going more and more into disfavor for three quarters of a century. Advances in surgery and, later, the promise of radiation seemed to be the answer. It appeared likely that these methods would end cancer as a serious problem. These techniques also helped to focus the prevailing medical view on cancer as a "local" phenomenon. The older organismic view was lost and the neoplasm was more and more considered as a problem of a specific body area, not of the body as a whole. All these factors apparently combined to reduce interest in the psychosomatic concept. Snow continued to write articles until 1904, trying to stimulate research, but he was unsuccessful.

[4] Ebstein, in 1894, for example, discussed the problem of the traumatic origin of leukemia. According to Hueper (*28*), Ebstein lists "psychic shock" as one of the possible traumatic factors.

As one reviews the literature up to this time, it is clear that these men quoted were highly experienced and competent cancer specialists who wrote not from a theoretical basis but on the foundation of years of careful observation. None were psychiatrists, and only one (Hughes) was a neurologist. Their technical tools were limited by the period in which they worked, and therefore they were more likely to observe the patient as a whole.

In the first quarter of the twentieth century, few comments on this concept were found by this writer. Mohr (*51*) suggested that there are psychically influenced, hormonal processes in carcinoma formation. Kehrer (*34*) stated his belief that psychological disturbances always exist prior to the formation of fibroma. In 1921, Willy Meyer (*48*) discussed his attempts to understand the relationships involved. He believed that cancer is often a result of social and emotional stress through the instrumentality of changes in the serum salts and blood chemistry. In his discussion, Meyer, who introduced radium therapy into America, showed the beginning of the trend from viewing cancer as a specifically local problem toward perceiving it again as a general problem of the organism whose most dramatic symptoms are in one area or another. It is only as this trend developed that a modern psychosomatic view of cancer became possible.

During this period, roughly 1900 to 1925, the field of psychoanalysis came into existence. This included a concept of the human psyche which made it possible to investigate many hitherto hidden facets of perception and response. The idea of using this concept, and the tools it made possible, to investigate diseases whose symptoms were organic, came early. Freud, in 1910, clarified the problem from the psychoanalyst's point of view in his paper "Psychogenic Visual Disturbance According to Psychoanalytical Conceptions" (*18*). Groddeck wrote widely on the subject (*25*) summing up his viewpoint in 1928 in *The Book of the IT* (*24*). In this, he conceived of cancer as being an "acting out" of deep frustrations on the part of the body. Today, few would accept Groddeck's extreme view, but even fewer would deny the stimulating and thought-provoking quality of his work. Smith Ely Jelliffe (*32*) pointed out the need for research in this field:

> I deem it absolutely necessary to emphasize that so far as the carcinoma problem is concerned, the facts are still too deeply buried to permit even speculative suggestions. It should be said, however, that ignoring the psychoanalytical method as part of the program of research for the ultimate understanding of carcinomatous and other malignant growths is stupid.

As the second quarter of the twentieth century opened, studies of the possible relationship between the psyche and the neoplasm began slowly to appear. The first of these was a brilliant and intensive study by Elida Evans (*12*). She studied 100 cancer patients by Jungian depth-psychotherapy. She reports that the typical cancer patient had lost an important emotional relationship prior to the development of the neoplasm, and was unable to secure any effective outlet for his psychic energy.

Under the stress of this intense renunciation, the detached energy had turned inward and, in expressing itself through a primitive erotic outlet, had brought the cancer into existence. She saw the basic problem of the cancer patient as that of being driven back into himself. Her book is thoughtful and provocative. One may disagree with the frame of reference of this serious scientist, but her data are very hard to dismiss.

This study provides an interesting comparison with two statements made from completely different orientations at about the same time. Hoffman (*26*, *27*) was a creative and perceptive statistician who had spent many years studying cancer as an epidemiological problem. His statistical studies led him to raise the question as follows:

> From the dietary disorders to nervous disorders is but a step. The two are frequently interrelated and cause and effect are often hopelessly confused. . . .This naturally brings me to the question of the interrelationship of cancer and worry, or the mental attitude of the patient toward the development, spread and curability of the disease. . . .My own investigations of more recent years have led me to accept much of the latter view as an aspect of the cancer problem well deserving of more careful attention (*27*).

Neither as a statistician nor as a psychoanalyst, but as an extremely sensitive physician, James Ewing came to this conclusion about the cancer patient:

> Many cancer cases are highly susceptible to mental suggestion and when many such cases get together and discuss their condition and testify to the relief from pain and increase of weight, certain physical effects of purely psychical origin may occur, which are not the result of the direct action of the agent employed (*13*).

These three approaches, psychological exploration, statistics, and clinical observation, illustrate the primary methods that have been used in recent research. As these techniques become integrated, we can expect more and more dependable information and deeper and more meaningful insight. Foque (*17*), in 1931, in a careful and important paper, stated his belief that there was a multiplicity of secondary causes for cancer, such as X rays, chemicals, and viruses. However, from his point of view, the cells had to be in a receptive state before the cancerous process could start. On the basis of his clinical experience, he believed it necessary to study "the role of sad emotions as activating and secondary causes in the activation of human cancers." These, he thought, through the instrumentality of the nervous system's effect on metabolism, act on the endocrine balances of the body in such a way that the cell is put into a state where it is sensitive and receptive to the carcinogen. "How many times have I heard, with variation due to different social statuses, the litany: 'Since the death of my child, doctor, I am not the same. I do not recognize myself. I cannot find my equilibrium, and that is certainly the beginning of my illness because before, nothing like this had come to my attention.' "

Foque did not take this too seriously at first, but when it occurred repeatedly and when he noticed that this type of emotion seemed to

accelerate the growth of the cancer, "I was led to consider with less incredulity and with scientific interest, the possible influences of these deep, sad emotions and their depressing psychology." He found, clearly apparent in many of his patients (one third of those with cancer of the stomach, and one fifth of those with cancer of the breast), ". . . great crises, grave depressive afflications, profound mourning, and all the sad emotions which have prolonged repercussions . . . you can see in the patients prolonged and silent sorrow without the release of sobs and tears." Foque believed that the death of a child is one of the strongest of these "grave, depressive afflications," which serve as an activating agent for cancer, but there are many others. In reading Foque's paper, one is startled by the similarity of his findings with those of the older writers. Indeed, an unmistakable stream runs through this entire literature: Gendron, Nunn, Parker, Paget, Walshe, Snow, Hughes, and many others, often obviously writing independently, use almost the same words in their descriptions. Again and again one comes upon the terms, "despair," "afflictions that appear unsurmountable to the patient," "hopelessness," "loss of a child," "loss of a spouse." In different countries and different times, these physicians looked at and listened to their cancer patients and found the same psychic symptoms.

LeShan and Worthington, in view of the fact that the loss of an important relationship prior to the appearance of the neoplasm had been reported by many observers, as well as in their own work (*37*, *39*), attempted to make a statistical test of this hypothesis (*38*). If this were valid, cancer rates in different social groups should vary with the likelihood that a strong relationship had been lost. For example, in different marital classes, the probability of the loss of a crucial emotional relationship would be highest in the "widowed" group; next in the "divorced" group; next in the "married" group (where the relationship with the spouse might have been lost, but the legal marriage was retained for religious, financial, or other reasons); and least in the "single" group. A prediction was then made that the age-adjusted cancer mortality rates would be highest among the widowed, next among the divorced, next among the married, and lowest in the single group. Various predictions of this kind were made. A survey of cancer statistics showed that in all cases where adequate statistics had been published, the predictions were borne out.

The effect of the loss of a spouse upon the development of cancer is itself amenable to statistical treatment. Using this method with expert care and precision, Peller, in a series of studies (*58*, *59*), was able to demonstrate that widows in all age groups had a higher cancer mortality rate than spinsters or married women. He was able statistically to rule out genetic predisposition, reproductive accomplishment, and social class as the important factors in this difference. "It is diminished resistance in widowhood that accelerates the appearance of and death from cancer" (*59*). "This circumstance in the carcinorelevant mechanism remains to be clarified" (*58*).

Other studies have been forced to the same conclusion. A typical

example is included in Murphy's study of heredity in uterine cancer (*52*). He carefully matched his probands and controls and found that:

> In the case of the cancer probands . . . 35.5 percent of the 184 marriages had been interrupted by either widowhood, divorce or separation before the time of the interview, whereas the marriages of only 16.4 percent of the controls had been so interrupted. There is no information available to explain this difference.

Studies by Lombard and Potter (*43*), Dorn (*8*), and others clearly indicate the same type of data. The clinical observations of the eighteenth and nineteenth century that the cancer patient had, more often than could be accounted for by chance, undergone "grave and depressing afflictions," is supported by the cancer statistics.

In 1948, Miller and Jones (*49*) reported on six cases of chronic myeloid leukemia they had studied. "These findings therefore, have led us to believe that the frequent occurrence of emotional difficulties in patients with leukemia may be more than a coincidental finding." Each of the six had a history of marked emotional stress arising before the leukemia was diagnosed, although they found no similar personality patterns among the different members of the group.

Leriche (*36*) sought the link between emotion and cancer in "deep metabolic disturbances, in a dysfunction of hormonal glands, in changed quantitative proportions between potassium, phosphorus, calcium and cholesterol" (*59*).

With the study by Tarlau and Smalheiser in 1951 (*68*), a new type of scientist moved into the picture. This was the clinical psychologist with his armamentarium of projective test and interview techniques. These are specialized clinical observation techniques that previous observers lacked. By and large, the new studies were done in complete ignorance of the older literature, *i.e.*, "Medical and psychological literature is remarkably free of studies of personality patterns in cancer patients" (*21*). In reviewing this literature, it will become apparent that the methods of the clinical psychologists brought to light much the same kind of data that the trained, perceptive, and intuitive eighteenth- and nineteenth-century oncologist had.

Tarlau and Smalheiser studied 22 women—11 with cancer of the breast and 11 with cancer of the cervix—by means of interviews, Rorschach tests, and drawings of the human figure. They found that the ". . . interview material and the Rorschach data gave consistent pictures for the two groups. . . . Both groups had similar family patterns of mother dominance which resulted in a rejection of the feminine role. Both groups had similarly negative attitudes toward sexuality, which contributed to sexual maladjustment." The cervix-cancer patients showed much more overt sexual disturbance than the breast-cancer group, who made superficial adjustment to their problems.[5] The cervix-cancer patients showed a higher incidence of overt marital discord. The interview material

[5] This study and several others consider premarital sexual relationships and adultery as indicators of poor sexual adjustment. One might raise the question of whether this is a value judgment or a scientific fact.

appeared to indicate that these differences were not a result of the cancer but had existed long before the first-noted symptoms.

Tarlau and Smalheiser caution that their study must be regarded as a preliminary one. The groups were small, and all the patients came from similar social backgrounds. In addition, all patients were in the terminal stages of cancer and had been ill for a long time. How much this influenced the data is not clear. In all probability, this study is more important for its pioneering quality of bringing forth new tools to cope with the problem than it is for the results reported.

Wheeler and Caldwell (*75*) included a control group in a replication of the Tarlau and Smalheiser study, and found that their data agreed on some points but not on others. However, "In the overall picture, the results of the present study do not point up striking differences between the three groups examined. In other words, the women are more alike than they are different."

Ferracuti and Rizzo (*14, 15*) studied a group of terminal-cancer patients, with a battery of projective tests. They found no consistent personality patterns in them.

Beatrix Cobb (*6*) reported on 100 cancer patients whom she had studied by means of projective tests, interviews, and questionnaires. She was primarily interested in the differences between those who came promptly for treatment and those who delayed. She found, however, some factors common to all her cancer patients. They regarded emotional involvements as dangerous. Her subjects showed more and stronger negative reactions to their families than were found in her control groups. Under stress, they tended to withdraw. ". . . as a group, they had difficulty making their way in a world of adequate social relationships."

Von Essen, in 1952 (*71*), stated that he felt that psychological exploration of cancer is probably of dubious value and may actually be dangerous since it may cause a physician to miss an X-ray diagnosis if he is looking for neurotic factors. However, he presented two case histories which forced him to consider this field in spite of these reasons.

Byron Butler studied a group of patients with malignant tumors, as part of a research on the value of hypnosis for the reduction of pain in cancer (*5*). He concluded in part, "There may be a 'cancer personality.' From a very intensive study of these cases, either an inhibited individual with repressed anger, hatred and jealousy, or a 'good' person consumed with self-pity may be the prototype."

Bacon, Rennecker, and Cutler obtained psychiatric case histories from 40 women with breast cancer (*3*), and found certain consistent behavorial characteristics. Thus, 35 of the women had a "masochistic character structure," 30 "had no techniques for discharging anger directly or in a sublimated fashion," and 25 had never experienced orgasm. Other consistent characteristics were "an unresolved hostile conflict with the mother, handled through denial and unrealistic sacrifice," and delay in securing treatment. The case histories of women who did not fit into this picture were usually in the oldest age group; almost all the younger women

had these characteristics. The basic problem of this younger group appeared to center around the unresolved conflict with the mother. The data in this paper (*3*) seem to have been obtained by highly trained psychiatrists and to have been handled with creativity and imagination. The lack of a control group raises certain objections, but the study is suggestive and important.

Stephenson and Grace made a comparison of 100 women with cancer of the cervix and 100 women with cancer of nonsexual sites (*67*). A medical and sexual history was obtained and an attempt was made to determine the attitudes of the patients toward their parents and toward other men and women. The data were considered "yes or no" material, and the two groups compared. Various indicators of sexual maladjustment appeared far more frequently in the cervix group than in the other group and "antedate the onset of the cancer by many years." This study must be regarded as a statistical research due to the manner in which the data were collected and handled. As such, it is stimulating and of value, but this is far from a clinical study of the personality of cancer patients.

Greene reported on 20 consecutive male admissions, ranging from 3 to 66 years of age, with a diagnosis of some form of lymphoma or leukemia (*21*). All were interviewed "at least once and some three or four times." Four adults were too sick to give much information, and the major data were obtained from friends or relatives. Data on the three children in the group were obtained from their parents. No control series was used. There are realistic questions about the validity of this type of material and at best, as Greene admits, "the study of these patients is superficial." In all 20, the development and recognition of the lymphoma or leukemia occurred while the patient was adjusting to a loss of support. This included the loss of significant relationships. In 17 patients, this loss involved varying degrees of separation from a parent-figure, usually the mother or a mother-figure. Frequently, this separation occurred when there was no available replacement.

In a continuation of this research, Greene, Young, and Swisher reported on 32 women with a diagnosis of lymphoma or leukemia (*22*). They conclude that ". . . one of the multiple conditions determining the development of a lymphoma or leukemia may be separation from a key object or goal, with ensuing depression . . . . It is inferred that all of these women had an unresolved attachment to their mother. Individually, they presented various personality characteristics and could be divided into four groups, designated as mothering, clinging, isolated and manly."

In their study of children with leukemia, Greene and Miller (*23*) found that "in 21 out of 23 children with leukemia, there was evidence of prodromal events which could be interpreted as separations or losses for the patient. . . . These findings suggest that in children, as well as in adults, separation with depression may be a necessary condition for the manifestation of leukemia."

Orbach, Sutherland, and Bozeman (*54*) report that, in their study of leukemic children, they were struck by the fact that the mothers of these

children showed a clear tendency to have disturbed relationships with their own mothers.

Dunbar, in her exhaustive survey of the psychosomatic field (*9*), views cancer as profoundly influenced by emotional conditions. She qualifies this, however, by stating that the relationship has not yet been proved. Hutschnecker (*30*) presents many case histories and clinical observations which have led him to see a clear connection between emotional states and the neoplastic process.

The work of West, Cutler, and Ellis (*see 20*) introduced a new facet into this field. They were impressed by the fact that it often appeared possible to make intuitive predictions as to how well a patient would respond to treatment, compared to other patients with similar clinical pictures, and that these predictions were borne out more often than could be explained by chance. They were unable to define the basis of their predictions.

> About the best that we could do was to say that those who got along well with cancer were often relatively "dull" and those who did not relatively "bright" individuals, but we were quite aware that this was a totally inadequate expression of what we had observed and certainly no more profound than the everyday sayings "too mean to die" and "too good to live."

In attempting to clarify the facts at issue, two types of personality tests were used: The Rorschach method, interpreted by Bruno Klopfer who is one of the outstanding experts on this test, and the Minnesota Multiphasic Personality Inventory. Patients were divided into three classes, based on their length of survival after the first-noted signs of the malignancy.

> Each neoplastic disease which is not cured by surgery or irradiation has a characteristic survival duration which is familiar to clinicians with experience in this specialty. From the great volume of statistics accumulated on this subject, some of the more comprehensive reviews were used as a guide. . . . Any surviving plus or minus 50 percent of the mean expected period were classified as average (*74*).

Those who died in the first quarter of the survival curve for their type of cancer were called "fast progressions." Those who survived into the last quarter of the survival curve were called "slows." In this way, three groups were differentiated: those who died quickly of the disease; those who died "average"; those who survived longest. The study only concerned itself with the fasts and the slows. The average group was not used.

West, Cutler, Ellis, and Klopfer set themselves the task of trying to predict into which classification a patient would fall. When the patient first appeared, he was given psychological tests. After the first group had been studied, and the course of their disease followed and compared to the psychological findings, predictions were made on a second group of patients. In this group of 50 patients with cancer, 39 predictions were accurate. The accuracy of these predictions was 78 percent: there was less than one chance in a hundred that this occurred by chance alone. The ". . . fast-growing cases may be described as having more defensiveness, a higher anxiety level, and less ability to reduce tension through motor discharge than the slow groups. . . .The more neurotic the defenses, the more

"Maginot-Line" psychology we find in an individual, the surer one can be that if he has a cancer, it will grow fast."

It is impossible to sum up briefly this very rich and provocative study. Comments by Ziskind, Clemens, Jacobs, and others, explore many of the questions raised. A clear demonstration seems to have been made that there is a correlation between those factors measured by personality tests and the growth rate of neoplasms.

LeShan and Worthington studied the personality of 250 patients with malignancies (*38*, *40*). They used short series of interviews, from 2 to 8 hours each, and a projective test, the Worthington Personal History. Ten patients were also studied intensively in over 1,400 hours of depth psychotherapy.

LeShan and Worthington reported a life-history pattern, which was found in 62 percent of their neoplastic subjects and 10 percent of their controls. This pattern included a childhood experience which made intense interpersonal relationships appear difficult and dangerous. When a strong, meaningful relationship was found and accepted, a tremendous amount of psychic and physical energy was poured into it. This cathexis became the center of the individual's life, his *raison d'etre*. He functioned adequately but superficially in other life areas. Then, for reasons beyond the individual's control, the relationship was lost. This occurred through such events as the death of a spouse, children growing up and moving away, retirement from a job, and others. Attempts to find substitute relationships failed, and the patient underwent a period of intense despair which was later repressed. Within a period of 6 months to 8 years after the loss of the relationship, the first signs of malignancy were noted.

In a continuation of this work, LeShan studied 20 cases of Hodgkin's disease (*41*). A special type of life-history pattern appeared in 18 of the 20 subjects, and atypically in the control groups.

Fisher and Cleveland (*16*) studied the relationship between the patient's psychological picture of his own body and the site of the cancer. One type of body-image visualizes the skin as a strong and protective barrier. An opposing type views the skin as permeable and easily penetrated. Fisher and Cleveland devised a method of scoring the Rorschach test, "the barrier score," which was based on these concepts. An attempt was made to predict from their Rorschach-test protocols whether patients had cancer on the exterior or the interior surfaces of the body. Blind analyses showed that this could be done with excellent validity and reliability. Evidence was presented that this differentiation in response was not due to the patient's symptoms, which might cause emotional focusing upon areas of the body, but to quite different factors. "We find the overall results obtained to be most meaningful if one assumes that an individual's unconscious image of his body is an index to aspects of his personality which can influence the site at which he develops physical symptoms."

Their study points up two different approaches to the effect of personality on the etiology of neoplastic disease. One approach groups all

malignant disease in a general syndrome with varied symptoms. Studies that fall into this class, *i.e.*, Evans, LeShan and Worthington, and Butler, do not differentiate particularly between patients with different types of cancer. Instead, they contrast "malignancy" and "nonmalignancy" groups. The second approach is to study patients with specific types of cancer, such as lymphomas, cancer of the breast, etc., and either contrast them with other types of cancer patients, as Tarlau and Smalheiser did in their study of cancer of the breast and the cervix, or else compare them to patients with no known malignancy. Studies in this class include those of Stephenson and Grace, Greene, Bacon, Rennecker and Cutler, and LeShan. At this stage in our knowledge, it cannot be said that either approach is the "valid" one or the other is "invalid." Obviously, both are necessary until we have more information and a better theoretical framework within which to formulate research problems.

The most sensitive and accurate method of exploring personality is through intensive psychotherapy. Very little has been done in this area, but there seems to be little question that future studies will include psychotherapy.

Kant (*33* and *see 11*) reported on a patient who presented severe "hypochondriacal" symptoms. These were cleared up by psychotherapy, but when the patient was to be discharged from the mental hospital, he suddenly died. Autopsy revealed a cancer of the pancreas with widespread metastases, which had clearly been responsible for the symptoms. Kant demonstrates by this example that reports by a patient, as to how he is doing, can follow an entirely different course from that of the physical disease. We are reminded of Von Essen's warning (*71*) on the dangers of observing psychological data at the cost of observing physical aspects of the problem.

Rennecker (*61*) described some of the psychotherapist's problems of emotional involvement when he engages in depth therapy with cancer patients. His descriptions are quite sobering and make it clear that a control therapist is absolutely necessary to any such program.

LeShan and Gassmann (*42*) discussed their findings after 1,400 hours of depth psychotherapy with patients who had fatal cancers. They indicate some of the special techniques needed, the emotional problems of the psychotherapist which are likely to arise, and the common psychological factors found in their cancer patients. In a section on the psychosomatic aspects of this type of therapy, they point out that changes in the growth rate of tumors are apparently highly correlated with certain types of emotional stress and with reductions in stress occurring in psychotherapy, and that this imposes the necessity for extreme care and often special methods on the part of the therapist.

The attempt has also been made to study psychological effects upon cancer by using animals. Macmillan (*44*) has briefly summed up some of the work of the Pavlovian school in this area:

> Petrova's work (1946) is reported by Ivanov-Smolensky (1954, pp. 34–38) and shows that: 1) cancers of the internal organs developed spontaneously

> in certain dogs subjected frequently and for long periods, to experimental neuroses; 2) that the possibility of carcinogenic substances producing skin cancers in both dogs and mice is realized only when experimental neuroses have been produced in the animals.
>
> Further experiments in the Kiev Oncological Institute have been reported by Kazansky (1955, p. 44), which show that natural or artificial sleep (both of which overcome functional derangements of the higher nervous activity) reduce the effect of carcinogens and transplanted malignant tumors.
>
> It is difficult not to conclude with Kazansky that ". . . functional overexertion of the central nervous system promotes, to a high degree, the appearance of spontaneous tumors, and also that of precancerous formations under the influence of carcinogens and the transplantation of cancerous tissues."

Reznikoff and Martin (*63*) compared the frequency of occurrence of cancer and its age of appearance in stressed and nonstressed mice.

> It was found that the mice without the predisposition to mammary cancer (milk factor) did not, in any instance, develop tumors even where exposed to intensive stress. More than three-quarters of the stressed animals with a susceptibility and a comparable number of their non-stressed controls, developed mammary cancer. However, the tumors tended to appear earlier in the stressed group of mice.

A study by Andervont (*2*) appears extremely suggestive in view of the frequency with which studies of human beings have indicated that loss of personal relationships is highly correlated with the appearance of neoplasms. Andervont raised C3H mice that showed a high percentage of spontaneous breast cancer under two types of conditions. The experimental group was first placed in group cages and then moved to isolation cages. The control group was kept in group cages. Statistically, the experimental group showed a significantly higher percentage of tumors which appeared at an earlier age. This was true for both virgin and breeding mice. (A more intensive study by Mühlbock (*50*) confirmed these results.) However, great caution must be taken with the entire animal approach. The dangers involved are clear in generalizing from animals to *Homo sapiens* in areas relating to personality. The analogies are often seductive and frequently misleading.

The pragmatic value of the research into relationships between personality and cancer is far from clear at the present stage of our knowledge. Psychotherapy, in itself, does not appear to be a useful therapeutic method. There will never be enough psychotherapists to deal effectively with the cancer problem. Further, the value of psychotherapy as a therapy for neoplastic conditions has never been established, in spite of some interesting and suggestive results. Even theoretically, the existence of a correlation between emotional stresses and cancer does not mean that easing of these stresses will halt the cancerous process. One does not put out a forest fire by extinguishing the match that started it. It is nevertheless true that it may be necessary, after putting out the fire with other means, *i.e.*, surgery, radiation, or chemotherapy, to extinguish the match in order to prevent a new fire. Certainly much research is needed on the

short- and long-term effects of changing emotional tensions when neoplasms develop.[6]

A major value in the study of the effects of personality stress on cancer may be in the clues to changes at different levels of biological function. Thus Trunnell (*70*) has reported his observations of cancer patients in multiple bedrooms of a hospital: "As the patient's friend in the next bed gets worse and dies faster than the man we're studying, almost without exception we see little increases in the serum acid-phosphatase level as the friend gets worse, and then when it's all over, our patient's acid-phosphatase level is fine again." The changes in this chemical level are accompanied by changes in pain and in the need for narcotics. This type of correlation is extremely suggestive and may lead to valuable insights.

In the writer's own experience, marked changes in psychological stresses have frequently been associated with sudden changes in the growth rate of tumors (*42*). There is evidence also that these stresses may be associated with changes in the excretion of sulfhydryls, in the surface tension and specific gravity of the urine, and with other changes. If we can determine that certain changes in body chemistry follow certain types of emotional change, and that there is also a correlation with neoplastic development, we may have new ways to attempt therapeutic intervention at different levels of biological function. It is probable that the greatest pragmatic value of research in this field will come from greater understanding of psychophysiological interactions rather than from purely psychological therapies.

We do not plan to discuss here the problem of the intervening variables between the psyche and the cell. At the present stage of our knowledge, this is a complex subject which deserves a longer exposition than space permits. Theories have ranged from Lobstein's concept of "perverted nutrition" (*72*) through studies of the adrenocortical axis (*70*) to Revici's concept of cancer as a hierarchically organized condition, with various pathogenic factors intervening at the different levels of the organization (*62*). Meerloo (*46*) and others (*69*) have discussed some aspects of this problem.

When a field is in its infancy as this one is, with many lines of investigation proceeding simultaneously, and before any over-all body of theory has been formulated, it is extremely difficult to sum it up briefly. In trying to evaluate the present situation in this area, certain factors appear to emerge:

1) There seems to be a correlation between neoplastic disease and certain types of psychological situations. This has been widely reported as a result of clinical observations, experimental studies, and statistical investigations.

2) The most consistently reported psychological factor has been the loss of a major emotional relationship prior to the first-noted symptoms of the neoplasm. It seems to be established that this has occurred more

[6] One example is verrucae: From the pathologist's standpoint, this is a benign tumor. The efficacy of suggestion as a therapeutic agent for the removal of warts is widely accepted.

often in cancer patients than in the various groups who were used as controls.

3) There appears to be some relationship between personality organization and the time from the first-noted symptoms of a neoplasm until the patient dies.

4) There may be some relationship between personality organization and the type or location of a neoplasm. The evidence that different types of people tend to develop different types of cancer is extremely suggestive.

The data and leads so far produced in this field seem worthy of much further investigation. This is an exciting and stimulating field which is bound to lead into many provocative problems, and may point the way to new means of approaching the neoplastic process.

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Effect of Carcinogenic Dosage and Duration of Exposure on Skin-Tumor Induction in Mice [1,2,3]

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Summary

Data are presented to indicate (a) threshold levels, for the induction of benign tumors and for their development to a malignant state, and (b) maximally effective carcinogenic dose levels of benzo[*a*]pyrene on mouse skin. The threshold dosage of carcinogen is apparently dependent in part upon chronicity of exposure. The reported findings indicate that continuation of the process of skin carcinogenesis is directly dependent on dose level and duration of carcinogenic exposure, as well as on potency of carcinogen. Experimental studies are discussed to support the concept that the malignant state in the process of carcinogenesis is a terminal development in a series of increasingly proliferative reactions of normal cells to a persistent physiologically irreparable state of tissue damage or of homeostatic imbalance. It is suggested that a carcinogenic agent is one that produces a physiologically irreparable state of damage or imbalance. The failure of carcinogen-induced neoplasms to progress to malignancy, if the causative factor is removed in time, re-emphasizes the need for identification and control of carcinogen-induced tissue damage and residual carcinogenic agents in man in order that a practical program of cancer control may be attained.—**J. Nat. Cancer Inst. 22: 19–43, 1959.**

Earlier studies of skin-tumor induction, reviewed by Stewart (*1*), Greenstein (*2*), Berenblum (*3*), Cowdry (*4*), and others, have demonstrated that the tumors induced with potent carcinogens may vary from hyperplastic epithelial proliferations, or papillomatous growths that regress soon after induction, to rapidly growing, invasive carcinomas. Tumors with these differing neoplastic growth potentials have been observed to appear sequentially or concurrently on the same animal. The large amounts of carcinogens employed in early studies were designed

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to yield high tumor incidences in the shortest period of time (*2*). This practice, however, tended to obscure such factors as dosage of carcinogen at levels below maximally effective ones and duration of carcinogenic exposure, as related to the type of tumor induced. Recent studies (*5*) have attempted to investigate both these aspects by employing a quantitative technique in applying the carcinogen benzo[*a*]pyrene to the skin of mice, and by extending the range of doses studied to levels below those previously reported effective (*6*). With this approach, dose-response relationships were ascertained in skin carcinogenesis, and the neoplastic response was shown to be a direct function of dosage when benzopyrene was applied repeatedly in μg. quantities (*7*, *8*).

Since the carcinogenic response depends in part on the intensity of chronic exposure, when dosage is regulated so that the biologic resistance of the host is not overwhelmed, it appears advisable to re-examine a number of current hypotheses on tumor genesis. Among these are the concepts that the ultimate evolution of the tumor is an all-or-none phenomenon (*3*), that the principal biologic characteristics of each tumor are probably determined at the moment of its inception (*9*), that there is no threshold dose of a carcinogen which can be considered harmless (*10*, *11*), and that tumor initiation involves an "instantaneous, irreversible, qualitative change (*3*)." A review of the regression of carcinogen-induced tumors in man and in experimental animals (*4*, *12–14*) raises doubts that the induction of the neoplasm involves an initial irreversible change, and warrants the alternate hypothesis that the summation of carcinogenic exposures determines the principal biologic characteristics of a tumor. Benign tumors of the skin, localized malignancies, and invasive epitheliomas may therefore represent consecutive stages in the course of carcinogenesis rather than different neoplastic entities whose characteristics are determined at the moment of initiation (*15*). Conversely, the process of skin carcinogenesis may be considered a multistage phenomenon with malignancy as the terminal stage of neoplasia (*15*, *16*). This concept is strengthened in the present report by data demonstrating the existence of threshold and maximally effective carcinogenic dose levels for benzo[*a*]pyrene on mouse skin and a relationship between the threshold dosage and chronicity of exposure.

Material and Methods

Mice.—C57L and DBA/2 mice from the Roscoe B. Jackson Memorial Laboratory and random-bred, Tumblebrook Farm, albino mice were used. All mice were housed in transparent plastic cages and given Purina chow and water *ad libitum*. Their routine processing after arrival in the laboratory included a month or more of isolation, during which time they were inoculated with vaccinia by tail scarification. Before the initial carcinogenic exposure, and periodically as needed, they were shaved in the interscapular area with electric clippers.

Carcinogen.—A single stock supply of commercial benzo[*a*]pyrene [6] of

[6] Hoffman-LaRoche, Inc., Nutley, N. J.

high purity was used. Solutions of 1.25 percent in toluene were prepared periodically on a weight-per-volume basis. Concentrations of 0.25, 0.05, 0.01, 0.005, and 0.002 percent were prepared by serial dilution from the 1.25 percent stock solution. The technique for quantified skin application of the carcinogen has been described (*7*): Each application consisted of a drop of one of the prepared solutions applied to the shaved interscapular skin with a blunted 20-gauge needle dropper. The needles were calibrated to deliver approximately 133 drops per cc. of toluene (*i.e.*, approximately 0.0075 cc. per drop). A thrice-weekly exposure schedule was maintained for the mice that required more than one application.

Experimental Procedures

The neoplastic effect of a single application of benzopyrene on male mice of the C57L strain, 18 to 20 weeks old on exposure, was studied. Five experimental groups, containing 11 to 14 mice per group, received 1, 2, 4, 8, or 32 drops of the 1.25 percent solution of benzopyrene in toluene on the shaved interscapular skin during a single exposure period. Each drop contained approximately 94 μg. of the carcinogen. Between successive drops the solvent was allowed to evaporate from the skin in order to limit the spread of the carcinogen. All mice that received less than 32 drops of the benzopyrene solution received the difference as additional drops of toluene. A control group of 7 mice received a comparable treatment which consisted of 16 drops of the solvent. All animals were kept for the remainder of their natural lives and observed for the development of tumors.

The effect of a varied number of applications of a carcinogenic dose level of benzopyrene on male DBA/2 and Tumblebrook albino mice, 5 to 9 weeks old on initial exposure, was studied. A dose level approximating 19 μg. benzopyrene per drop of 0.25 percent solution in toluene was used, in view of the excessive carcinogenic potency for repeated doses in larger amounts. Exposures consisted of 1 drop of 0.25 percent solution on the mice in 1 group, thrice-weekly applications of 1 drop for a total of 10 or 20 drops on the mice in 2 other groups, and 60 or more drops (*i.e.*, until a grossly malignant-appearing tumor had developed) on a fourth group. In the last-mentioned group, the induced tumors ranged from 3 to 10 mm. in diameter at termination of exposures. To ascertain the residual effect of carcinogenic exposures in the absence of an induced neoplasm, 1 group of DBA/2 mice received 20 applications beginning when they were 8 to 9 weeks of age and a second course of thrice-weekly applications after they were 60 weeks old. The second course was continued until malignant-appearing tumors were observed.[7] As in the preceding study, the mice were inspected for the development and growth of tumors throughout the remainder of their lifetime after initial exposure.

The effect of chronic applications of benzopyrene, in the dose range of 0.15 to 752 μg. per application, on the C57L strain, was studied. Thrice-

[7] A gross criterion for malignancy was arbitrarily chosen to be the development of a tumor approximately 6 or more mm. in diameter, with subcutaneous thickening of the base and rolled or infiltrating margins.

weekly drop applications of a benzopyrene solution in the concentration range 0.002 to 1.25 percent were made to the interscapular skin throughout the experimental lifetime of the mice, or until a grossly malignant-appearing skin tumor had developed.[8] The calculated amounts of benzopyrene so deposited were 0.15, 0.38, 0.75, 3.8, 19, and 94 μg. per drop. Dose levels of 188, 376, and 752 μg. were obtained by applying 2, 4, or 8 drops of the 1.25 percent solution of benzopyrene during each exposure period. Control mice received equivalent drop applications of the stock supply of toluene.

Gross examination for the detection of tumors was made thrice weekly when the mice were treated. Further examination under magnification was made every 4 or 5 weeks, and the development of gross neoplastic or other pathologic lesions was charted individually for each mouse. Histologic preparations were made of the principal organs and interscapular skin of all animals at the end of their natural lifetime except for the few that were lost because of *post-mortem* autolysis or cannibalism. Tissues were fixed in buffered formalin, embedded in paraffin, and stained routinely with hematoxylin and eosin.

Results and Conclusions

The results are summarized in tables 1 to 6.

Effect of Single Exposure—Experiment 1

Fluorescence persisted from 1 to 3 weeks after exposure, in the interscapular skin of mice that received a single application of 94 to 3008 μg. benzopyrene. This suggested that the carcinogenic effect extended considerably beyond the instant of application. Papillomas were induced on only 12 mice; of these, the growths on 8 subsequently regressed[9] (table 1). The regression of carcinogen-induced changes of the skin, such as acanthosis and hyperkeratosis, and the regeneration of some hair follicles, obliterated by the high-dose, single-exposure treatment employed, are represented in figures 1 and 2. Two of the 4 persistent papillomas induced at the higher dose levels progressed to a state of low-grade malignancy and were found terminally to be slow-growing squamous-cell carcinoma.

The percentages of animals with a gross neoplastic response elicited by a single dose applied to the skin show that the incidence of induced tumors is directly associated with the magnitude of dose applied. The results confirm the work of earlier investigators who induced skin tumors in a fraction of their mice after a single massive exposure to a highly potent carcinogen (*17*, *18*). In addition, these data suggest that the induction of a transient benign tumor, the induction of a benign but persistent tumor,

[8] *See* footnote 7.

[9] In an earlier experiment, a group of mice was treated with benzopyrene in comparable fashion. Approximately 30 minutes after treatment each mouse was swabbed with toluene and a mixture of lanolin and mineral oil in order to remove as much benzopyrene as possible. None of the mice developed tumors during a 5-month observation period. This experiment had to be terminated subsequently because of an epizootic.

TABLE 1.—*Neoplasms induced by single application of benzo[a]pyrene on mice*

Benzo[*a*]pyrene in single application* (*μg.*)	Number of mice treated†	Number of mice responding	Number by type of neoplasm		
			Transient papilloma	Persistent papilloma	Epidermoid carcinoma
None	7	0			
94	14	1 (7%)	1		
188	14	1 (7%)	1		
376	13	2 (15%)	1	1	
752	13	3 (23%)	2		1
3008	11	5 (45%)	3	1	1

*Solution of 1.25 percent in toluene applied dropwise to shaved interscapular skin of C57L male mice, 18 to 20 weeks old, approximately 94 μg. per drop.

†Mice that survived at least 20 weeks on experiment, or until the first tumor was induced within the experimental group, if that occurred in less than 20 weeks.

and the development of a malignancy are, in part, dose-dependent phenomena, and that threshold dose levels of exposure exist in carcinogenesis.

Effect of a Varied Number of Exposures—Experiment 2

The neoplastic effect of repeated exposures to the dose level 19 μg. benzopyrene in toluene is shown in tables 2 and 3. Neither Tumblebrook nor DBA/2 mice manifested grossly apparent neoplastic responses to one application of the carcinogen. Tumblebrook mice that received 10 or 20 applications subsequently developed papillomas during their lifespan; the slightly more resistant DBA/2 mice showed only a minimal response to 20 applications. None of the papillomas so induced were progressive in growth, nor was there other gross or histologic evidence of an induced epidermoid carcinoma in or adjacent to the interscapular skin. In contrast, all mice of both strains that received 60 or more applications developed tumors that progressed to epidermoid carcinoma (table 2). Figures 3 to 8 illustrate induced malignancies in one of the DBA/2 mice that

TABLE 2.—*Incidence and type of tumors induced by benzo[a]pyrene* applied thrice weekly to shaved interscapular skin of mice†*

Number of exposures	Number of mice treated‡	Number of mice with induced tumors	Type and number of induced tumors		
			Transient papilloma	Persistent papilloma	Epidermoid carcinoma
1	8	0 (0%)			
10	8	5 (62%)	3	2	
20	8	7 (88%)	5	2	
72–117§ DBA/2	8	8 (100%)	‖	‖	8
20	7	1 (14%)	1		
60–100§	11	11 (100%)	‖	‖	11

*Approximately 19 μg. applied dropwise as a 0.25 percent solution of benzopyrene in toluene.

†Tumblebrook Farm, random-bred albinos.

‡Mice that survived at least 20 weeks on experiment or until the first tumor was induced in the experimental group, if that occurred in less than 20 weeks.

§Until the induced tumors were 5 to 10 mm. in diameter.

‖Induced but not tabulated.

TABLE 3.—*Cumulative effect of repeated carcinogenic exposures**

	Group 1 (4 mice)	Group 2 (11 mice)	Group 3 (7 mice)
I. Duration of initial series of applications.	7 wks.	7 wks.	7 wks.
II. Number of weeks between initial and secondary course of treatments.	52 wks.	Second course followed immediately after first.	No second course.
III. Duration of secondary treatment until tumors were induced on each mouse.	6, 8, 8, 17 wks.	6, 12, 12, 16, 17, 18, 21, 21, 21, 21, 21 wks.	—
IV. Duration of secondary treatment until tumors appeared grossly malignant.	21, 12, 23, 17 wks.	21, 13, 15, 21, 21, 21, 21, 21, 21, 27, 27 wks.	—
V. Number of mice with epidermoid carcinoma at death/number mice effectively exposed.	4/4	11/11	0/7

*Approximately 19 μg. benzo[*a*]pyrene in toluene applied dropwise thrice weekly to shaved interscapular skin of DBA/2 male mice, 8 to 9 weeks old.

responded with a metastasizing squamous-cell carcinoma and a locally invasive fibrosarcoma.

It is again apparent, from table 2 as from table 1, that the induction of benign tumors may be a consistently reproducible consequence of exposure to a carcinogen, and that the induced tumors may regress or persist but apparently do not proceed to malignancy if exposure to the causative agent is interrupted before a threshold for carcinoma genesis is exceeded. These data also support the impression of a direct association between the duration of repeated exposure and the incidence and degree of malignancy, *i.e.*, the invasiveness of the induced skin neoplasm. It is again apparent that the process of carcinogenesis is directly dependent on dose level and duration of exposure as well as on potency of carcinogen.

As seen in table 3, a first carcinogenic exposure results in an accelerated neoplastic response to a second course of treatment even after a 52-week interval between exposures. Twenty treatments with individual doses of 19 μg. benzopyrene, applied to DBA/2 mice in a period of 7 weeks, induced no tumor during the lifespan of the control animals (group 3). As in experiment 1, there was no persistent gross or histologic evidence of irreversible malignant change due to this initial set of treatments. Acanthosis and hyperkeratosis of the interscapular skin became less distinct as the interval from the initial exposure period increased, while hair-follicle regeneration did take place in depilated areas before the second course of treatment was resumed after a delay of 52 weeks. Continued thrice-weekly treatments without interruption after the first 7 weeks induced papillomas in a second group of mice (group 2) after 6 to 21 weeks of additional treatment. Continuation of the initial treatment in

a third group of mice, after a 52-week period had elapsed between treatments (group 1), induced papillomas within 6 to 17 weeks after treatment was resumed. In the latter two groups there was no significant difference either in the time of tumor appearance or in the development of a grossly malignant-appearing state relative to the total duration of exposure.

These data confirm similar findings of Lavik, Rusch, Cramer, Berenblum, and others (*2*), and support the concept that one treatment of the skin with a carcinogen may "sensitize" the tissue to subsequent applications. However, in view of a better understanding of mechanisms of sensitization since those experiments were reported, the interpretation that "dormant cancer cells are produced on initial exposure" must be reconsidered.

Effect of Chronic Exposure—Experiment 3: Summary

The effect of lifelong, thrice-weekly, repeated applications of an individual dose from 752 to 0.15 μg. benzopyrene is seen in tables 4 to 6. Depending on dose level of repeated application, gross neoplastic manifestations ranged from transient papilloma induction in a few mice at the low-dose level of 0.15 μg. to epidermoid carcinoma induction and curtailment of the expected lifespan in all mice subjected to the higher doses, 19 to 752 μg.

1) *Dosage and time of tumor appearance.*—The time elapsing between the beginning of chronic exposure and the appearance of a definite skin tumor suggests different rates of reaction to the carcinogen, which are dependent upon dosage or intensity of exposure (table 4). The most rapid reaction rates were within the groups exposed to 752, 376, 188, 94, and 19 μg. per application: their median tumor-appearance time showed a stepwise prolongation, from 9 to 19 weeks, associated with decreased dosage. With the exception of 2 mice in the 188 μg.-exposure group, which died before the group's median tumor-appearance time, all animals within this dosage-span developed an early neoplastic response. The earliest appearance of a neoplasm at the 3 highest dose levels shown in table 4 was masked on many mice by crusts of hyperkeratotic debris and caked benzopyrene. As a consequence, in a few instances, the tumors were large enough to be classed as malignant when they were first noted.

The mice subjected to applications of 3.8 μg. benzopyrene showed a more delayed reaction. Of the adequately exposed mice in this group, 84 percent developed a neoplasm. Their median tumor-appearance time was 37 weeks as compared with 19 weeks for the nearest, higher dose level (19 μg.).

In contrast, mice exposed to 0.75, 0.38, and 0.15 μg. per application showed a third level of reaction. Within this range, only a small number in each exposure group developed neoplasms, usually late in their experimental life. At these low-dose levels, mice died of old age and related factors, with papilloma incidences of only 9 to 20 percent after exposures up to 103 weeks.

The response of the last group, in some respects, provides an experi-

TABLE 4.—*Skin-tumor induction with benzo[a]pyrene in toluene applied thrice weekly until malignant tumor induction or death of C57L mice**

Benzo[*a*]-pyrene applied thrice weekly (*μg.*)	Number of mice with induced neoplasm		Median (Range) (*weeks*)	Tumor appearance time (*weeks*)†																		
	Ratio	Percent		1–56–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45	46–50	51–55	56–60	61–65	66–70	71–75	76–80	81–85	86–90	91–95	96–100	101–105
0. 00	0/33	0																				
0. 15	5/55	9	(42–62)								2	1		1	1								
0. 38	11/55	20	(24–103)				1					1	3	1		1	1					1	2
0. 75	7/56	13	(36–60)							2	1	2		2									
3. 8	41/49	84	37 (21–58)				6	4	10	9	7	3	1	1									
19. 0	38/38	100	19 (11–25)		8	15	15																
94. 0	35/35	100	17 (8–22)		2	5	23	5															
188. 0	12/14‡	86	15 (9–20)		1	6	5																
376. 0	14/14	100	11 (4–19)	2	4	3	5																
752. 0	13/13	100	9 (5–19)	1	6		6																

*Nine to 19 weeks old on initial application.
†Tabulation includes all induced tumors: those that remained benign and those that became malignant.
‡Two mice died without tumors after 6 and 12 weeks of exposure.

TABLE 5.—*Skin-tumor induction by benzo[a]pyrene applied thrice weekly until malignant tumor induction or death of C57L mice**

Benzo[*a*]pyrene in toluene (*μg.*)	Number of mice with epidermoid carcinoma		Median	Gross malignancy appearance time (*weeks*)†														
	Ratio	Percent		Range and distribution														
				1–5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45	46–50	51–55	56–60	61–65	66–70	71–75
0. 15	0/55	0												1				
0. 38	2/55	4										1		2	1			
0. 75	4/56	7						1		2	4	8	3	10	1	3		
3. 8	32/49	65	41				1	22	8	4	2							
19. 0	37/38‡	97‡	25			2	8	22	2	1								
94. 0	35/35	100	21			1	4	5										
188. 0	10/14‡	71‡	19		1	1	6	4										
376. 0	12/14‡	86‡	19		2	3	8											
752. 0	13/13	100	19															

*Nine to 19 weeks old on initial application.

†Duration of exposure until an induced tumor was at least 6 mm. in diameter with a subcutaneously thickened base and rolled margins.

‡Death of mice that did not have histologically demonstrable epidermoid carcinomas occurred before their groups' median time for gross malignancy appearance (*cf.* table 6).

mental analogy to the environmental cancer experience of man, for it is only a small segment of the human population-at-large that develops a neoplastic response after being exposed repeatedly to trace amounts of environmental carcinogens; the neoplasm is not always malignant, and it appears rather late in life.

Acceleration in tumor-appearance time with increasing doses of benzopyrene from 1 to 100 μg. per application was reported in previous communications (*5*, *7*). As an extension of that finding, these data show that the chronic exposure period for tumor appearance is progressively shortened by increasing doses at least up to 376 to 752 μg. per application. The association of variations in tumor-appearance time and tumor incidence with dosage of the carcinogenic agent re-emphasizes the significance of dosage as a determining factor when estimating comparative activity of different agents or when attempting to pinpoint an etiologic factor for a neoplastic response with a prolonged incubation period.

2) *Dosage, malignancy-appearance time,*[10] *and lifespan.*—The potency of the various dose levels for the production of epidermoid carcinomas and their effect on lifespan may be gauged from tables 5 and 6. A few mice in the groups that received 752, 376, 188, 94, and 19 μg. per applied dose died prematurely.[11] All others developed skin tumors that became malignant. It is apparent that, under highly carcinogenic-exposure conditions for the species, biologic differences, due to strain, age, sex, etc., modified the carcinogenic response only quantitatively by influencing the induction time of the initial neoplasm, during the early portion of the animal's experimental lifespan, and its rate of growth to a malignant state. Biologic variability under these conditions did not prevent a neoplasm from developing on any chronically exposed survivor. This observation has now been confirmed in work with seven different strains (*19*).

At the dose level of 3.8 μg. (0.05% benzopyrene in toluene) 66 percent of all exposed animals developed carcinomas. This was the closest approximation, obtained experimentally, to a 50 percent carcinoma-inducing dose for chronic exposure. At 0.38 μg. per application, only 4 percent, *i.e.*, 2 of 55 mice developed carcinomas and they were among the 11 mice that had previously developed papillomas (table 4). With 0.15 μg., no malignancies were induced despite lifelong applications (table 5), although 5 of the 55 mice in the group developed papillomas. Thus, the carcinogenic threshold dose for chronic exposure of this strain was probably not more than 0.15 μg. At the other extreme, the similar effects obtained in the groups exposed repeatedly to 752 and 376 μg., *i.e.*, regarding the range of time required for initial tumor appearance (table 4), the median time required for development of a grossly malignant-appearing state (table 5), and the brevity of their median lifespan (table 6), suggest that a maximally effective carcinogenic dose level had been approximated.

[10] *See* footnote 7.

[11] A premature death at any dose level of exposure was so classified if it occurred before the median tumor-appearance time for the group, provided the dosage was adequate for tumor development in at least 50 percent of the animals.

TABLE 6.—*Lifespan in relation to neoplastic response and dose level of benzo[a]pyrene in toluene applied thrice weekly*

Benzo[*a*]pyrene applied thrice weekly (*μg.*)	Number of C57L mice treated	Experimental lifespan							
		Epidermoid carcinoma		Benign skin neoplasms		Mice without gross skin neoplasms		Entire group	
		Range / Median (*weeks*)	Incidence (*percent*)	Range / Median (*weeks*)	Incidence (*percent*)	Range / Median (*weeks*)	Incidence (*percent*)	Range / Median (*weeks*)	
0.00	33					29–92 / 60	100	29–92 / 60	
0.15	55			44–66 / 62	9	16–98 / 60	91	16–98 / 61	
0.38	55	82, 83	4	24–103 / 69	16	19–99 / 58	80	19–103 / 65	
0.75	56	58–67 / 62	7	57, 61, 64	6	15–94 / 51	87	15–94 / 56	
3.8	49	42–82 / 61	65	25–59 / 46	18	25–65 / 40	16	25–82 / 56	
19.0	38	21–44 / 39	97	25	3			21–44 / 39	
94.0	35	19–43 / 28	100					19–43 / 28	
188.0	14	18–35 / 28	72	21, 21	14	6, 12	14	6–35 / 21	
376.0	14	20–35 / 23	86	15, 16	14			15–35 / 23	
752.0	13	13–30 / 23	100					13–30 / 23	

The early appearance of tumors during the experimental animal's lifespan under high dosage intensities may be contrasted with the delayed manifestations of neoplasia and the broader range of biologic variability at what may be called threshold levels (about 0.15 μg.). At the threshold levels there is no neoplastic response among the majority of exposed animals, but in the more susceptible minority there is a development of transient or persistent noninvasive tumors with limited growth potentials; the lifespan of the animals is not significantly curtailed (table 6). In fact, the median lifespan at the two lowest dose levels is slightly longer in the experimental group than in the control group.[12]

Discussion

From the data in each of these experiments, it is apparent that the benign or malignant characteristics of a tumor, as well as its induction time, are partly dependent on the factors of carcinogenic dosage, duration of exposure, and the relative potency of the carcinogen. It is also apparent that threshold levels for carcinogenesis can be ascertained for each of the three types of exposure employed, and that intense or highly concentrated doses may be tolerated without gross neoplastic sequelae, provided the total duration of all exposures is proportionately brief in relation to the potency of agent and dosage, and recurrent exposures are minimized. Conversely, the demonstrated dependence of threshold dosage on chronicity of exposure emphasizes the hazard of chronic intermittent or continuous exposure to environmental carcinogens.

These experimental findings have their epidemiologic counterpart: The indication that threshold and subthreshold conditions of carcinogenic exposure exist for man is found in the fact that most human deaths are not due to cancer despite prolonged intermittent exposure of the majority of mankind to trace quantities of chemical, physical, and biologic carcinogens in the environment. Even within industrial or other environmental areas, recognized to be hazardous from a carcinogenic point of view, the incidence has been relatively low compared to that customarily obtained in the experimental laboratory. Yet, within such areas, the high incidence of malignancy among those chronically exposed to high-dose intensities leaves little doubt that dose-duration-response relationships govern tumor induction for man as well as for the mouse.

The present findings suggest the advisability of re-evaluating a number of hypotheses related to the pathogenesis of neoplasia. The temptation has been strong and persistent to look upon carcinogenesis as a process initiated by a somatic mutation (*21*) or by the sudden, irreversible conversion of normal cells to a neoplastic state (*3, 9, 11, 12, 22*). Probably the most popular of these concepts, the "two-stage theory of carcinogenesis," suggests that the second stage, "promotion" of tumor-cell multi-

[12] Although more data on this finding are desirable, the results obtained provide an interesting similarity with the effect of radiation on lifespan. Upton has reviewed the evidence that the lifespan of mice is shortened in proportion to the rate at which radiation is administered. However, at very low doses (0.1 r per day of γ-rays throughout life) the experimental results were equivocal, with irradiated mice exhibiting a slightly longer mean survival time than the nonirradiated controls, despite a significantly higher incidence of certain neoplasms (*20*).

plication and growth of the tumor to grossly detectable proportions, may be effected either by continued exposure to a carcinogen or repeated stimulation by any one of a hypothetical class of agents, "cocarcinogens" or "promoting" factors, which by themselves need not be carcinogenic. Warburg's theory, so effectively refuted by Weinhouse (*23*, *24*), was an attempt to explain the undefined "irreversible" malignant change in biochemical terms. Allied to the concept of instantaneous tumor initiation resulting from exposure to a carcinogen are the ideas that inherent, qualitative differences exist between "benign" and "malignant" tumors, that a malignant tumor, contrasted with one that is benign, develops by a progression that is characteristically inexorable, and that the principal biologic characteristics of each tumor, from the time of the first irreversible, progressive, neoplastic change, are probably determined at the moment of its inception (*3*, *9–12*, *21–23*, *25*).

There is little if any adequate substantiating experimental evidence for these concepts except early reports that skin-tumor induction follows a single application of relatively large amounts of a potent chemical carcinogen in high concentration (*17*, *18*, *26*). In such instances no attempt was made to limit the exposure time of the carcinogen on the skin. In our own experiments, fluorescence of the exposed skin has been observed to persist for more than a week after a single application of benzopyrene, and comparable fluorescent periods have been observed with the more potent agents, 9,10-dimethyl-1,2-benzanthracene (DMBA) and methylcholanthrene (MCA), on mouse skin. Apparently, the "single exposures" of past experiments were in effect exposures of extended duration. The extrapolation from the works of earlier investigators that a single exposure led to an instantaneous, irreversible conversion to malignancy appears therefore to have been entirely unwarranted. The same considerations apply to any "single" administration of carcinogen (urethan, etc.), since the time of exposure in the organism is undetermined.

Radiation has been considered the universal carcinogen, and the most potent mutagen. Of all experimental carcinogens, radiation is the one to which exposure can be truly instantaneous. Yet, even in the area of radiation biology, there are no convincing data that the initial stage of carcinogenesis for any tissue involves the instantaneous production of malignant cells (*27*, *28*). In at least one instance, *i.e.*, the induction of tumors of endocrine glands, it is now generally accepted that a direct, mutagenic radiation effect is of little if any significance (*13*, *28*). A long-lasting state of hormonal imbalance is recognized to be the specific essential for tumor development in an endocrine gland as it is for tumors in endocrine target organs (*28*). Actually a long-lasting state of physiologically irreparable tissue damage appears to have been the specific essential for experimental tumor development since Yamagiwa and Itchikawa first produced epidermoid carcinomas by patiently and repeatedly applying tar to the ears of rabbits.

The concept has been proposed that, through differences in their biologic or neoplastic development, tumors induced by croton oil, presumably a

cocarcinogen or promoter, might be differentiated from tumors induced by a "low dose" of a carcinogenic hydrocarbon. This is based, at best, on observations of carcinoma induction in more than 50 percent of mice exposed to a 0.01 percent solution of DMBA when compared with the preponderance of benign papillomas and a high rate of papilloma regression in mice treated repeatedly with croton oil (*3*, *11*). The inappropriate designation "low dose," of a level of exposure that induces carcinomas in more than half of the exposed animals is probably another indication of the ingrained belief in a qualitative difference between the genesis of benign and malignant tumors, in the belief that cells activated by a carcinogen are irreversibly converted to malignancy and that the malignant course of a carcinogen-induced tumor is determined at the time of its inception (*3*, *9*, *11*, *12*, *21*, *22*, *25*). Current observations, on the contrary, continue to show no valid experimental basis for assuming that tumors induced with threshold amounts of a chemical carcinogen are in any way different from those due to "cocarcinogenic" or "promoting" agents. Evidence that cocarcinogens or promoters are in effect carcinogens of low potency, or that they contain potent agents in high dilution, has been reviewed previously (*8*). Parallel observations of others support this finding, and indicate that carcinogen-induced tumors may regress just as "cocarcinogen"-induced tumors may become malignant (*29*).

The era of experimental carcinogenesis includes among its highlights: demonstrations of radiation-induced carcinomas in rats by Clunet (1910), viral-induced malignances in fowl by Rous (1911), studies on exogenous chemical carcinogenesis by Yamagiwa and Itchikawa (1915), studies on hormonal carcinogenesis by Leo Loeb (1919) and Lacassagne (1932), the later studies by Rous and coworkers, Berenblum, and others, who developed the "two-stage" concept of carcinogenesis; and, in the present period, the demonstrations of plastic carcinogens (*30*, *31*) and "polyoma viruses" (*32*). A common denominator of all these investigations has been the demonstration that tumor induction results from prolonged contact of a body tissue with a carcinogenic agent or from persistence, over an extended period, of physiologically irreparable tissue damage. A multistage sequence of carcinogenesis, in which reactive hyperplasia is elicited as an early manifestation, and malignancy as a terminal development, is therefore an experimentally supported concept, with no need for unproved assumptions of initially irreversible changes, instantaneously induced latent tumor cells, cocarcinogens, and never-demonstrated inherent differences between "benign" growths and potentially malignant ones.

Further insight into the process of carcinogenesis may be adduced from 1) the reports of tumor development around implanted chemically inert plastic disks or sheets (*30*, *31*), 2) the reports of skin tumors arising outside a chemically treated area (*17*, *29*), and 3) the observations that presumably normal, nonirradiated autologous or indigenous cells and untreated, implanted donor cells may give rise to malignancies in an irradiated host (*13*, *33–35*). That tumors develop many months after the introduction of a sheet of chemically inert plastic, through which reticular

or connective-tissue cells cannot interlace to form cellular bridges, is probably an ideal example of a minimally traumatizing, yet physiologically irreparable, condition within the body, which eventually elicits a malignant growth. The chemical inertness of synthetic plastics supports the assumption that the reactive cells from which the malignancy arises are the undamaged, or only slightly affected, normal cells of the host. These experimental observations suggest the advisability of reconsidering a limited version of the chronic irritation and trauma theory as the fundamental cause of cancer. Granted that the nonspecific forms of this concept are untenable, the conclusion is inescapable that persistent, irreparable tissue damage or a persistent state of physiologic imbalance, which is beyond the adaptive capacity of the unaided organism, is an essential precursor of the malignant state. A tentative working hypothesis may therefore be suggested for laboratory validation: Carcinogenesis is an extreme form of reactive hyperplasia to a persistent, physiologically irreparable state of tissue damage or homeostatic imbalance, resulting ultimately in malignant overgrowth of cells related to the impaired tissue through contiguity or homeostatic mechanisms. Implied in this hypothesis are the concepts that the carcinogen is an agent which is capable of producing a nonlethal, yet persistent, physiologically irreparable state of cell or tissue damage, and that the malignancy consists of host cells which have reacted to the tissue damage or homeostatic imbalance, rather than to the carcinogen.

If the reported experimental findings and this tentative proposal have a bearing on areas of cancer prophylaxis and therapy, it may be in the implications *a*) that the initial neoplastic reaction to a carcinogen involves the induction of premalignant changes or benign tumors; *b*) that carcinogen-induced tumors do not necessarily progress to malignancy if the causative agent and/or its persistent damage are removed, in time, from the environment of the affected tissue.

In view of the findings in experimental carcinogenesis, it does seem reasonable to emphasize at least two working areas: *a*) the detection, identification, and neutralization of residual carcinogens in the organism, which may bring on a malignant condition and remain in the host to nullify therapeutic efforts by inducing recurrences; *b*) the detection and identification of the carcinogen-induced premalignant state, which we believe exists in one or another form for every malignant process. Through greater emphasis on prophylaxis, the need for therapy may thus be reduced, and the probability of its success increased.

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PLATE 1

FIGURE 1.—Interscapular skin of a C57L mouse 14 weeks after a single application of approximately 752 μg. benzo[*a*]pyrene in toluene. Acanthosis is evident. The bulk of a thick keratinized layer was detached from the skin and is not shown in the illustration. Skin appendages are completely absent. The pigment in the subcutaneous tissue is probably from the phagocytized remains of the degenerated hair follicles. × 325

FIGURE 2.—Interscapular skin of a C57L mouse 35 weeks after an equivalent exposure, showing regression of carcinogen-induced changes. Hyperkeratosis is prominent, but acanthosis is markedly diminished. Some hair-follicle regeneration is evident. × 325

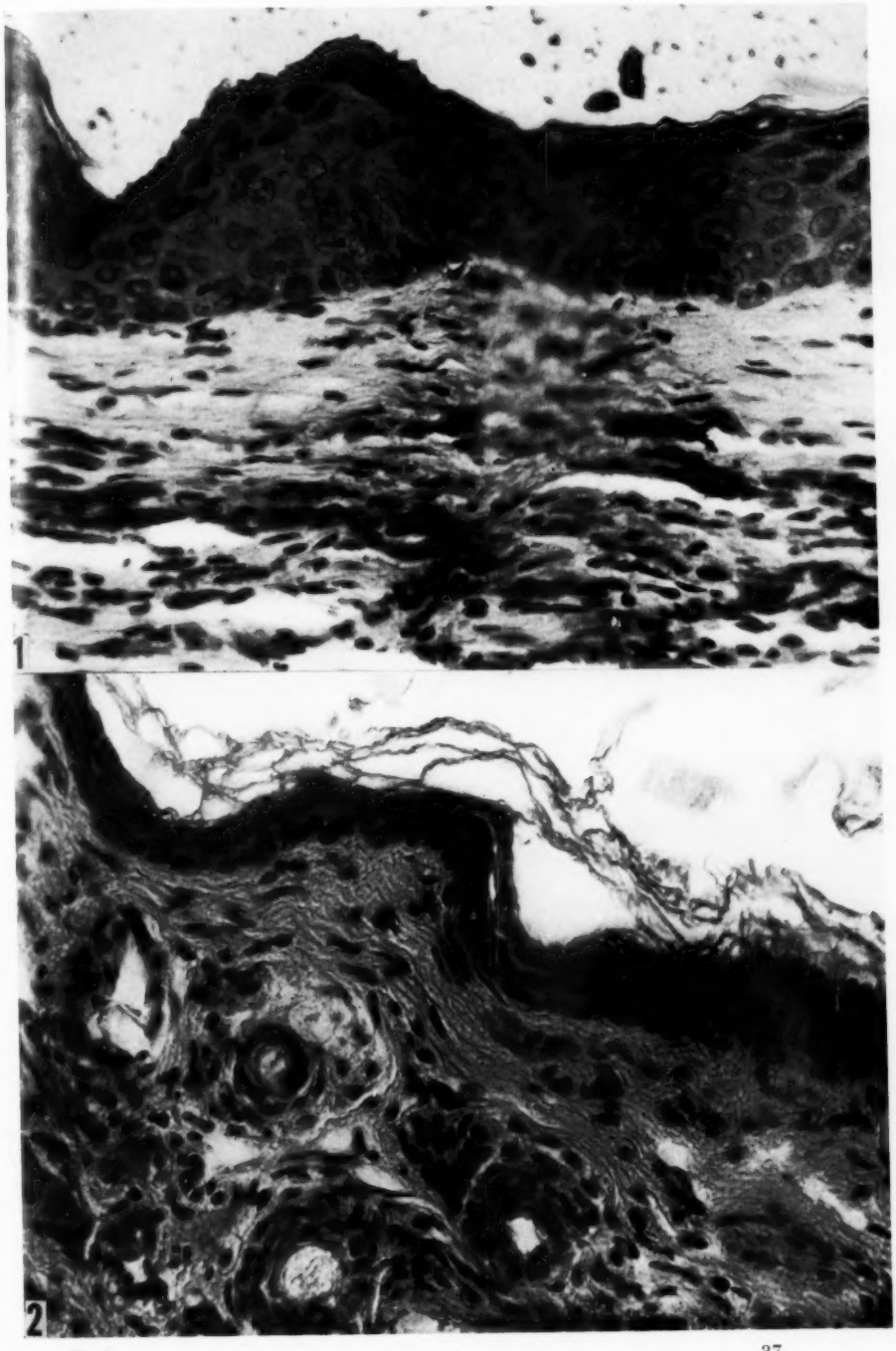
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PLATE 2

Thrice-weekly repeated applications of approximately 19μg. benzo[*a*]pyrene in toluene induced no malignancies on the interscapular skin of DBA/2 mice when exposure period was limited to 7 weeks. Prolongation of exposure period to 20 weeks or more resulted in squamous-cell carcinomas on all exposed mice.

FIGURES 3 and 4.—Squamous-cell carcinoma and fibrosarcoma induced within the interscapular region of a mouse after 20 weeks of repeated exposures to 19 μg. benzo[*a*]pyrene.

Poel

PLATE 3

FIGURES 5 and 6.—Metastasis of carcinoma to the lungs and cervical lymph nodes.

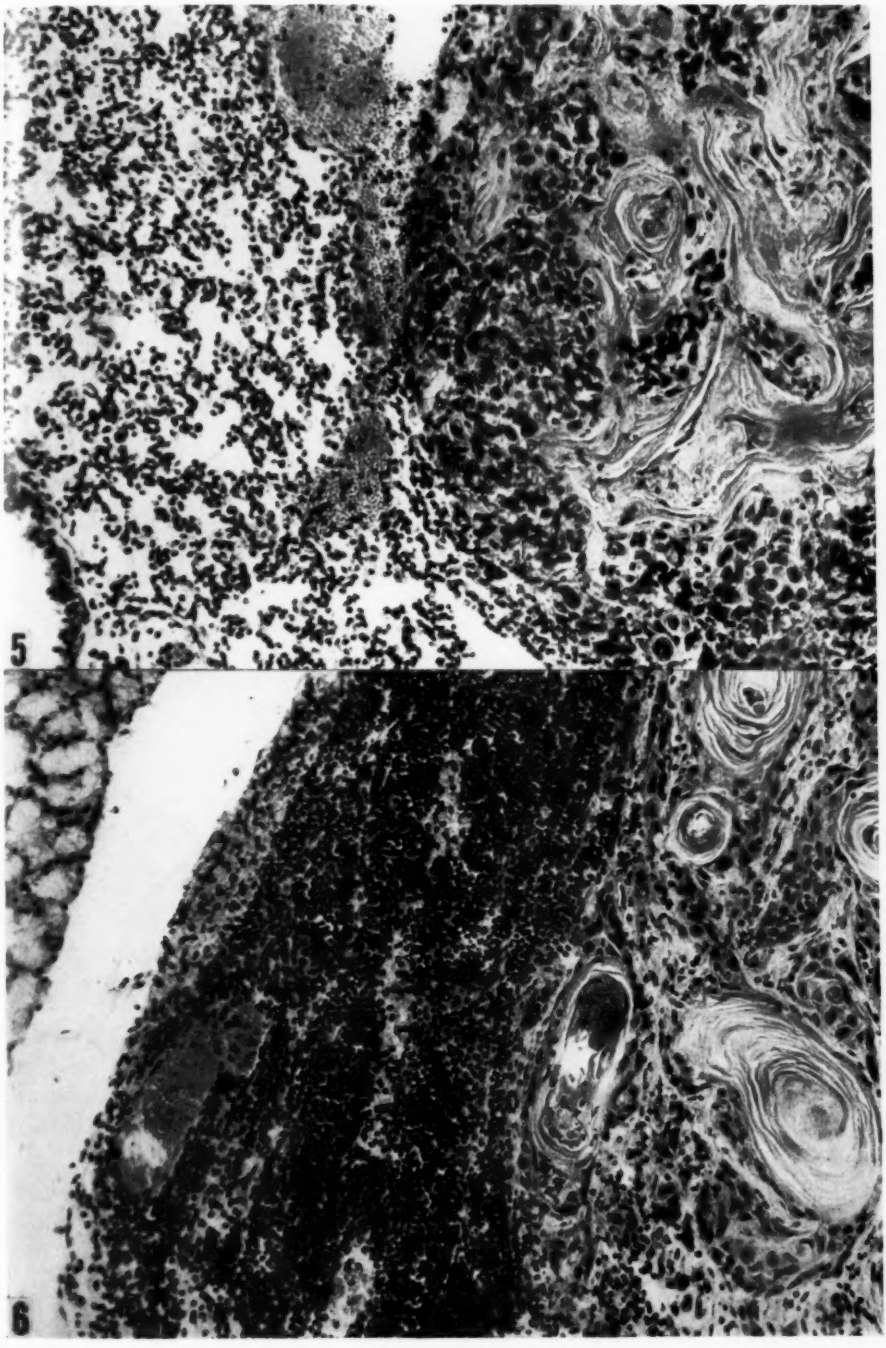
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PLATE 4

FIGURES 7 and 8.—Junctional area of primary tumors, showing sarcoma infiltrating and absorbing carcinoma, leaving residual islands of keratin.

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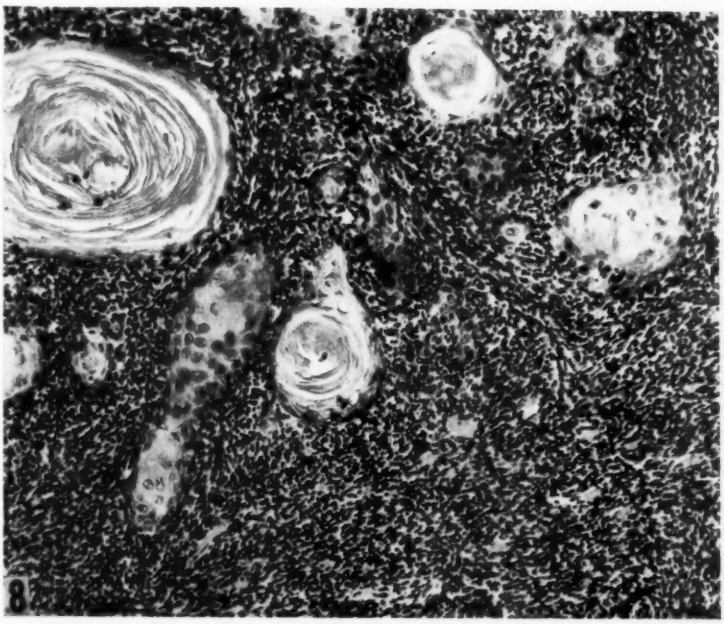
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Immunoelectrophoretic Characteristics of Normal Mouse Serums as a Basis for Studying Pathological Changes in Serums of Mice Carrying Transplantable Malignant Growths [1,2]

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Summary

With the technique initially developed by Grabar and Williams, which was modified by Scheidegger and further modified by Heremans, an immunoelectrophoretic investigation of serum from normal (CBA × DBA/2)F_1 hybrid mice is described, with special reference to the β- and γ-regions. More than 20 specific precipitation lines can be distinguished in the whole-serum electrophoresis, 9 of which belong to the β- and γ-regions. Some characteristic features are mentioned for each of these lines.—**J. Nat. Cancer Inst. 22: 45–55, 1959.**

Since Grabar and Williams (*1*) introduced the method of immunoelectrophoresis, a tool has become available for a more detailed study of serum-protein fractions. It not only allows the resolution of the conventional electrophoretic peaks of human serum into many individual components, but also appears to be an appropriate method for the study of minute, qualitative, structural abnormalities of each component.

The application of the immunoelectrophetic method to the investigation of human myelomatous proteins was first made by Grabar and coworkers (*2*). They found that, in practically every case, 2 of the normal serum proteins were specifically involved in the process of the disease. One, the γ-globulin, often showed a considerable, but very localized, increase that represented the conventional "paraprotein peak," and usually was associated with a reduction of the uninvolved part of the γ-component. Almost all "gamma" myelomas, but also some "M" and "beta" myelomas, appeared to derive their paraprotein from corresponding electrophoretic regions of the normal γ-protein.

The second protein involved in human myeloma disease was first observed in tracings obtained from normal human serum and was called

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[3] Holding a scholarship from Købmand i Odense, Johann og Hanne Weimann, f. Seedorffs Legat, Copenhagen, Denmark.

[4] The authors are indebted to Miss C. Gillant and Miss M. Th. Delcon for their technical assistance.

β_{2A} (*3*, *4*). It was recently isolated from normal human plasma by two of us (*5*) and was shown to be a glycoprotein with pseudoglobulin properties; it has some antigenic relationship to normal γ-globulin. In human myeloma disease it may be either considerably increased and qualitatively abnormal, giving rise to some of the β-paraproteins mentioned in the literature, or it may almost completely vanish.

The results obtained by immunoelectrophoresis in human myelomas stimulated us to apply this technique to the study of the abnormal protein fractions observed in the serums of mice bearing transplantable plasma-cell leukemias (*6–11*). Short preliminary reports on two of these plasma-cell leukemias have been given (*6–8*)—one associated with the development of a β-paraprotein, the other with an abnormal protein fraction in the area between the β- and γ-range (M-type). Recently, a more detailed account of the origin of these two plasma-cell leukemias and numerous other plasma-cell leukemias in (CBA $\times$ DBA/2)F_1 hybrid mice has been published (*12*).

As no immunoelectrophoretic studies appear to have been undertaken on mice, we found it necessary to trace the immunoelectrophoretic pattern of serum from normal (CBA $\times$ DBA/2)F_1 hybrid mice. This paper is concerned with a description of the technique used in this and further investigations and also gives an account of the various normal protein fractions which may be individualized by means of immunoelectrophoresis.

Materials and Methods

Immunoelectrophoresis.—The immunoelectrophoresis was performed according to the micromethod described by Scheidegger (*13*) and modified by one of us (*14*). Briefly, microscope slides covered with 2 ml. of Difconoble-agar gel (2%) in 0.025 N Veronal buffer, *p*H 8.6, were used as a supporting medium. Filter-paper connections led from the gel into electrode vessels containing 0.050 N Veronal buffer of similar *p*H. The electrophoresis was run in a moist chamber of Plexiglas®(methacrylic acid) for 3½ to 4 hours at approximately 4 volts per cm., and at room temperature.

Usually 2 μl. of undiluted or diluted serum was deposited into holes made in the gel by a needle with an internal diameter of 0.8 mm. After the electrophoresis had been completed, the tracing could be either fixed and stained for the development of protein areas or submitted to diffusion against an antiserum that was introduced into a 0.8 mm.-broad slit, running parallel to the electrophoretic migration direction for about 6 cm. The distance between this antibody reservoir and the hole from which the electrophoresis had started was usually 3.5 mm.

If the conventional protein pattern was desired, the agar electrophoretic slide was fixed for at least 1 hour in an aqueous, 4 percent acetic acid solution, dried at room temperature, and stained with Amidoschwarz 10 B, according to the method of Uriel and Scheidegger (*15*), except that a 50:10:40 mixture of water, acetic acid, and ethanol was used for decoloring.

Immunoelectrophoretic precipitation patterns were allowed to develop in a moist chamber for about 24 hours. The slides then were washed in frequent changes of 0.9 percent NaCl containing 0.1 percent NaN_3 as an antiseptic, for at least 48 hours, and again washed twice, for at least 1 hour, with distilled water, after which they were dried and stained with Amidoschwarz 10 B, in the manner described.

Antiserums.—Different antiserums against normal F_1 hybrid mouse serum were obtained from rabbits.

Antiserum L-50 was produced by an animal that had received 4 intramuscular injections of 0.5 ml. of mouse serum at 6-week intervals. The first injection was associated with Freund's antigen, and the animal was bled to death 1 week after the last injection.

The other antiserums, 229, 2-HV, and 1-GR, were obtained by a 6-week injection schedule comprising 2 weekly intramuscular injections of 0.2 to 0.4 ml. of F_1 mouse serum.

All antiserums were concentrated 4 to 5 times by the following procedure: The serum globulin fraction was isolated by salting out with either 2 M ammonium sulfate or 2.1 M phosphate buffer, *p*H 6.6; the redissolved proteins were then concentrated by pressure or suction dialysis against either a Veronal buffer or a 0.9 percent solution of NaCl.

It was noticed that L-50 contained a considerable amount of anti-γ-antibody but only little antibody against the other proteins of the same region, except for one of the β-globulins. On the other hand, 1-GR developed weak antibodies against numerous β-globulin components. Therefore it seemed advisable to prepare an antiserum showing intermediate properties, which was composed of 5 parts 1-GR and 7 parts L-50 and was called *BDK*.

Results

Plate 5 shows a confrontation of a conventional paper electrophoresis and its scanning diagram, a conventionally stained agar-gel electrophoresis, and an immunoelectrophoresis obtained from the same pooled, normal (CBA $\times$ DBA/2)F_1 hybrid mouse serum.

Three main protein fractions can clearly be recognized both on paper and on conventional agar electrophoresis. They may conveniently be termed *albumin*, *alpha*, and *beta* peaks. A faint zone lying cathodically with respect to the β-region can also be distinguished on the paper strips and is sometimes visible on agar tracings, and deserves the name of γ-globulin. When using larger amounts of serum on thick #3 Whatman filter paper, a *prealbumin* is also found, and on some of the tracings it is possible to see additional bands occupying the intermediate zones between the main peaks.

The use of terms such as α, β, and γ to describe the mentioned regions is justified by a comparison that was carried out with tracings from normal human serum.

The immunoelectrophoretic tracing shown in figure 3, plate 5, was obtained with BDK antiserum and illustrates the fundamental correspond-

ence between the conventional peaks and the pattern of the precipitation lines. At this point it must be recalled that each serum protein upon diffusing away from its initial electrophoretic localization builds a precipitation line where it encounters its corresponding antibody in optimal proportions. It follows from the laws of diffusion that each line assumes the form of a bow whose curvature, intensity, and distance from the antibody reservoir are determined by both the antigen and the antibody concentrations. Thus if there is an excess of one of the two reactants, the bow is displaced in the direction of the other reactant and *vice versa*.

It is known that in the tracings of human serum, 3 bows, γ, β_{2A}, and β_{2M} are not symmetrically curved but extend for some distance along the axis of the electrophoretic migration. This is commonly interpreted as indicating a heterogeneity with respect to the electrophoretic mobility associated with common antigenicity of all molecules of each component. It can be seen in figure 3 that the line representing the mouse γ-globulin has similar characteristics.

It is further obvious that several proteins have their precipitation lines centered upon areas lying between the main fractions. This justifies the introduction of dividing the α- and β-regions into several subgroups, so that the following nomenclature of mobilities is proposed: prealbumin, albumin, α_1, α_2, β_1, β_2, and γ. In this way we tried to come as near as possible to the currently used terminology of Grabar and coworkers for human serum, but the conventional character of the proposed designations is of course fully realized.

Plates 6 and 7 show the immunoelectrophoretic development obtained on normal (CBA $\times$ DBA/2)F_1 hybrid serum by using our 5 antiserums, 229, 1-GR, 2-HV, L-50, and BDK, respectively.

On each slide the most polyvalent of these antiserums, 1-GR, was used as a reference in the central graft. The diffusion was allowed to proceed for 36 hours instead of the usual 24 hours in order to allow the growth of the lines up to the fusion of identical lines from the lateral reservoirs with those of the graft filled with 1-GR.

For each electrophoretic region the bows were labeled after their relative mobility within the region, starting from the most anodically to the most cathodically situated protein. The mobility of the proteins corresponding to each bow could easily be deduced from the point where the bow came nearest to the slit. As an example, a system of designations, such as $\beta_{2\text{-I}}$, $\beta_{2\text{-II}}$, $\beta_{2\text{-III}}$, etc., was used for the β_2-region.

It appeared that only the proteins of the γ- and β-regions were of interest for the study of plasma-cellular mouse serums. Therefore this paper will deal only with the description of the bows in these regions. A more detailed description of the whole tracings, as well as some chemical characterizations of a few proteins and a study of their correspondence with known human proteins will be published later.

In the β_1-region, which in our experimental conditions is centered around the filling hole, only one protein line could be distinguished so far. We are calling it $\beta_{1\text{-I}}$.

The β_2-region possesses as a landmark a large bow visible with most antiserums, which corresponds to the protein that builds up most of the staining of the β-region. The latter point is proved by the fact that this bow, which we have named $\beta_{2\text{-I}}$, is the last one of the region to disappear when progressive dilutions of the F_1 serum are submitted to electrophoresis. The figures show that antiserum 229 develops $\beta_{2\text{-I}}$ very forcefully (plate 7); antiserums 1-GR and BDK develop it with moderate strength (plate 6); and antiserum 2-HV almost lacks the corresponding antibody (plate 7).

The next β_2-bow, which may be called $\beta_{2\text{-II}}$, is visible with all antiserums. It seems to correspond to some β-globulin of rather low concentration, since it is always weakly developed.

The third bow is $\beta_{2\text{-III}}$, which is only clearly seen with L-50, and less distinctly with BDK and 229. The antibody titer against this protein seems to be exceedingly low in 2-HV and 1-GR. As will be shown in a following paper (*16*), this protein appears to be of considerable interest for the study of plasma-cell neoplasias.

The fourth β_2-bow is only visible with 1-GR and 2-HV. The nature of this bow will be discussed in a later paper.

The bows lying more cathodically than the β_2-region, though not as far as the slowest γ-globulin, are called β_3. Only the first of them, $\beta_{3\text{-I}}$, is visible with all antiserums, though it is usually weak. It can best be seen with antiserum 2-HV, and presents a remarkable feature in that it really consists of two bows. The faster bow is sometimes very difficult to distinguish from the faster part of the γ-globulin line, but is very clearly seen with 2-HV. The slower part of the bow serves as a landmark for the β_3-region.

The two other β_3-lines, $\beta_{3\text{-II}}$ and $\beta_{3\text{-III}}$, are very weak and only distinctly shown by 1-GR antiserum. The antiserum 2-HV demonstrates also one β_3-line, possibly, $\beta_{3\text{-III}}$.

The slowest of all proteins is termed γ-globulin. As in human serum (*3*), its bow really extends much farther anodically than could be guessed from a mere inspection of the conventional paper tracing. It can sometimes be followed up to the fastest β-region. This feature probably indicates electrophoretic heterogeneity, as it does in human serum. However, in normal mouse serum only the slower half of the γ-line is clearly developed, while the faster part usually resolves itself into a cloudy streak, which indicates much less antigen in that region. It is best developed by antiserum L-50. We do not know yet how to interpret the remarkable splitting of the γ-bow which may be observed with BDK antiserum. Perhaps the composite nature of the latter is responsible for this peculiarity.

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PLATE 5

Normal, pooled (CBA × DBA/2)F_1 hybrid mouse serum.

FIGURE 1.—Conventional paper-strip electrophoresis.

FIGURE 2.—Conventional agar-gel electrophoresis.

FIGURE 3.—Immunoelectrophoresis developed with antiserum BDK.

FIGURE 4.—Scanning diagram of bromphenol blue-stained paper strip.

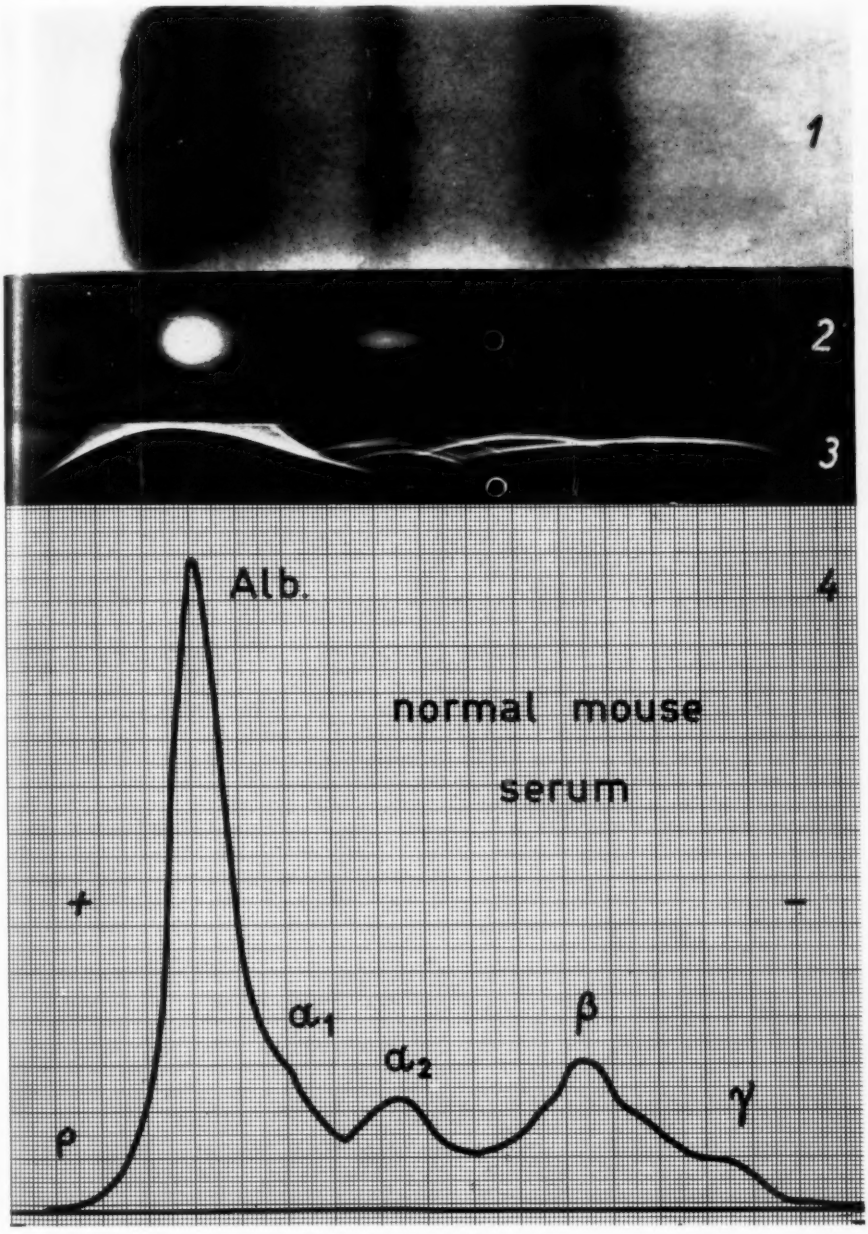

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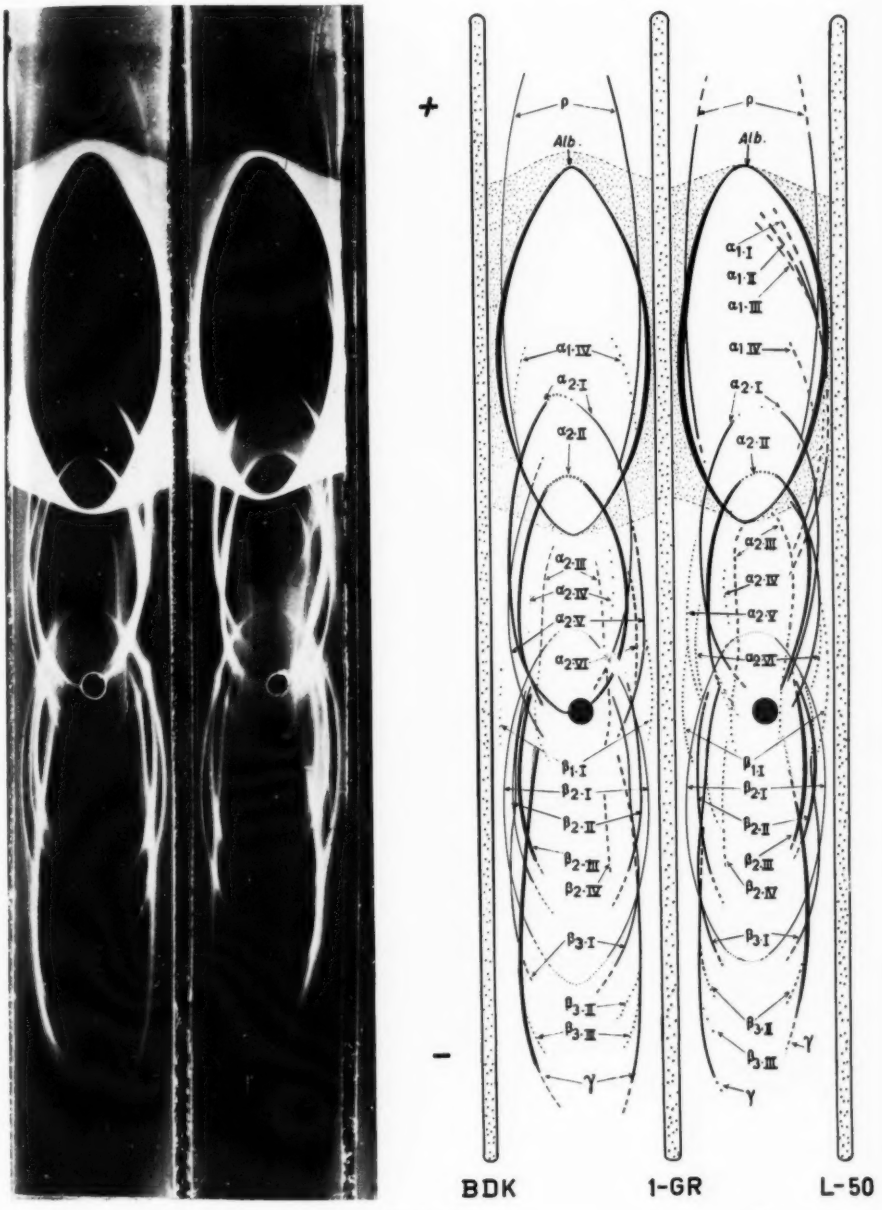
PLATE 6

Normal, pooled (CBA × DBA 2)F_1 hybrid mouse serum.

Left side: Photo of immunoelectrophoresis developed by means of 3 different antiserums; from the *left*, BDK, 1-GR, and L-50.
Right side: Drawing of the immunoelectrophoresis with labeling of all developed precipitation lines.



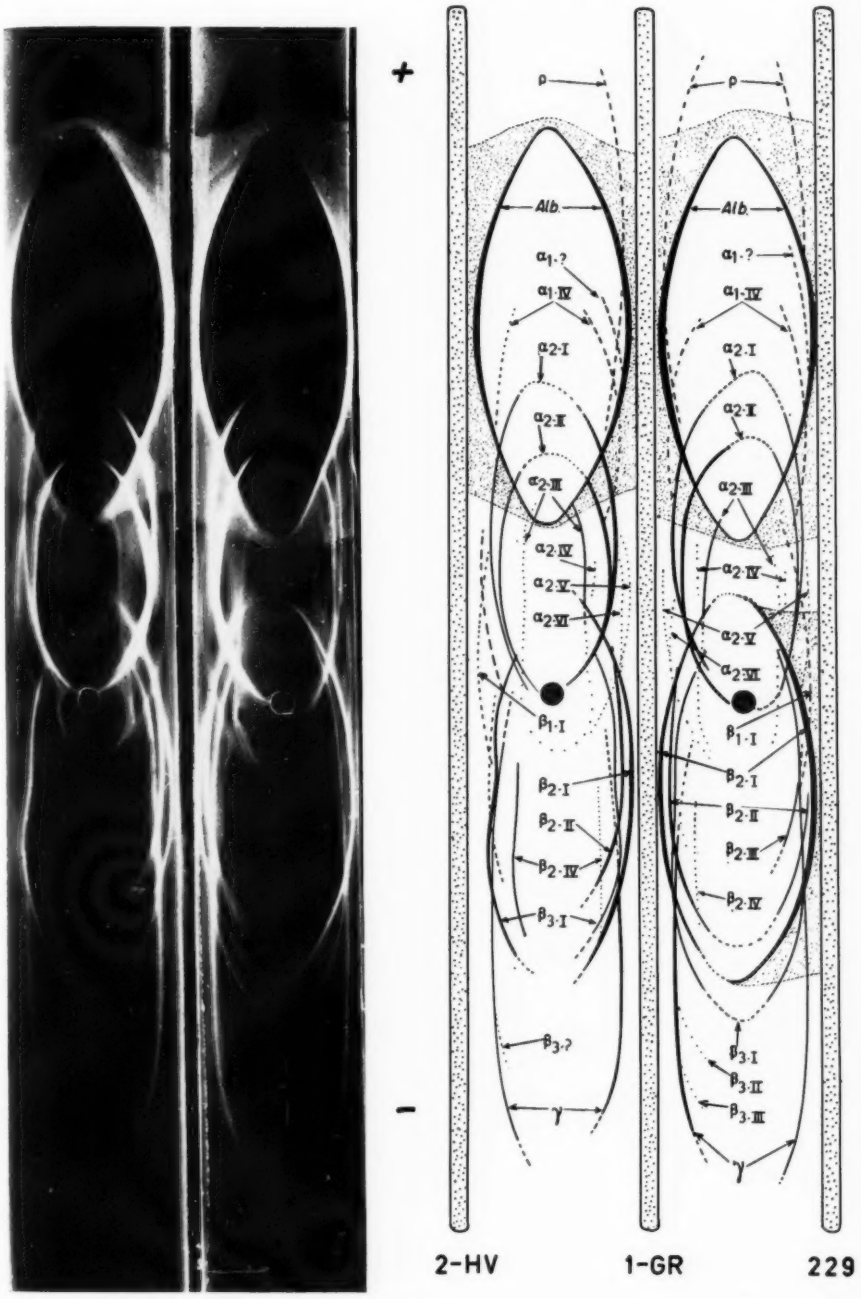
Heremans, Clausen, Heremans, and Rask-Nielsen 53

488412—59——7

PLATE 7

Normal, pooled (CBA × DBA/2)F_1 hybrid mouse serum.

Left side: Photo of immunoelectrophoresis developed by means of 3 different antiserums; from the *left*, 2-HV, 1-GR, and 229.
Right side: Drawing of the immunoelectrophoresis with labeling of all developed precipitation lines.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α2-V
α2-VI
β1-I
β2-I
β2-II
β2-III
β2-IV
β3-I
β3-?
γ
2-HV
1-GR
229

Immunoelectrophoretic Studies of Serums from Mice Carrying Two Transplantable Plasma-Cell Leukemias [1,2]

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Summary

Immunoelectrophoretic investigations were made on serums from mice carrying 2 plasma-cell leukemia *vid.* transplantation lines 20 and 36 associated respectively with development of β-paraprotein and γ-paraprotein with M-mobility. In line 20, the β-paraprotein-developing leukemia, this abnormal protein fraction immunoelectrophoretically belonged to the faster part of the normal mouse γ-globulin, thus labeling this leukemia immunochemically as γ-type with β-mobility. Another abnormality was increase and faster mobility of $\beta_{2\text{-III}}$-globulin, and increase of $\beta_{2\text{-I}}$-fraction. Line 36 divided in the first transfer into 2 sublines, 36 y and 36 x, with and without γ-paraprotein of M-type, respectively. The serological characteristics within each line remained unchanged for several passages. In paraprotein-containing serum from line 36 y, significant increase in the intermediate part of the γ-globulin bow was seen. In serums from line 36 x, this γ-bow was fainter than in normal serum. In line 36 y, the $\beta_{2\text{-III}}$-globulin showed the same increase and abnormal velocity found in line 20. Increase in $\beta_{2\text{-I}}$-fraction was more pronounced than in line 20. In line 36 x the same abnormality of $\beta_{2\text{-III}}$, but no increase in $\beta_{2\text{-I}}$, was detected. In all 3 transplantation lines, parts of the γ-bow not involved in paraprotein transformation were reduced in concentration. The significance of changes observed in $\beta_{2\text{-III}}$- and $\beta_{2\text{-I}}$-fractions and the correlation of these changes to the leukemic process is unknown. The findings were discussed and were compared to electrophoretic patterns seen in human beings with myelomas.—**J. Nat. Cancer Inst. 22: 57–81, 1959.**

The occurrence of plasma-cell leukemia in mice was first reported in 1951 (*1*). Since then other reports of transplantable plasma-cell neoplasias of the mouse have appeared in the literature. Bichel (*2*), Dunn (*3, 4*), Potter *et al.* (*5–7*), and Rask-Nielsen and Gormsen (*8–10*), independently, observed such diseases in various strains of mice and described the pathology and clinical manifestations.

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[2] These investigations were supported by grants from Civilingeniør H. Jacob Nielsens Legat, Dansk Ingeniørforening, Denmark, and from The Leukemia Society, Inc., New York, U. S. A.

[3] Holding a scholarship from Købmand i Odense, Johann og Hanne Weimann, f. Seedorffs Legat, Copenhagen, Denmark.

[4] The authors wish to express their gratitude to Miss C. Gillant and Miss M. Th. Delcon for their technical help.

488412—59——8

The striking similarities between human myeloma disease and these neoplasias prompted Potter *et al.* to study the serum protein pattern in plasma-cell tumors in C3H mice. Two transplantable plasma-cell neoplasms were studied; one showed serum changes, the other did not. Other transplantable reticular neoplasms were also studied for changes in serum proteins; no abnormalities were found.

Rask-Nielsen, Clausen, and Gormsen also demonstrated abnormal protein fractions in serums from (CBA × DBA/2)F_1 hybrid mice carrying transplanted plasma-cell leukemias. Preliminary reports on 2 transplantation lines that gave rise to "paraprotein" patterns in the serum have been issued (*9–11*). One line (transplantation line 20) was constantly associated with a paraprotein peak in the β-region of the serum electrophoresis on paper strips. Simultaneously a considerable increase in the carbohydrate content of the β-region was observed, thus labeling the abnormal protein fraction as a glycoprotein (*11*). This finding is strongly reminiscent of human myeloma disease, in which the β-paraproteins are notoriously rich in carbohydrate (*12*, *13*). The second transplantable plasma-cell leukemia (transplantation line 36) was accompanied by a similar paraprotein whose mobility was distinctly slower than that in line 20. It was described as a γ-myeloma protein of M-type, and was found to be a glycoprotein as judged from periodic acid-Schiff-stained paper electrophoresis strips (*11*). A preliminary study was undertaken to show the possible correlations between histological and cytological data and the paraprotein type (β or γ). No relationship has been established so far (*9*, *10*) but these investigations are still in progress.

Since the recently introduced technique of immunoelectrophoresis, first developed by Grabar and Williams (*14*), allows the resolution of the conventional electrophoretic patterns into many individual components, we found it appropriate to use this method for further elucidation of the serological changes observed in our plasma-cell leukemias.

The present paper deals with the immunoelectrophoretic findings in serums from mice carrying the transplantation lines 20 and 36. For comparison, the immunoelectrophoretic pattern of normal (CBA × DBA/2)F_1 hybrid mice was investigated and described in a preceding paper (*15*).

Materials and Methods

Experimental animals.—Both transplantation lines discussed in this paper arose in (CBA × DBA/2)F_1 hybrid mice; their origin together with that of numerous other plasma-cell leukemias has recently been described (*16*). Detailed descriptions of the serological, hematological, and morphological findings in these two lines and in other transplantable plasma-cell leukemias associated with the development of γ-paraprotein are under preparation.

Transplantation line 20.—This line originated in a 27-month-old female with splenomegaly and enlarged lymph nodes. On transplantation into other (CBA × DBA/2)F_1 hybrids it manifested itself as a rather slowly

developing leukemia. The survival time in the first passage was about 15 months; in later transfers about 2 to 5 months. The leukemia has now been carried through 8 passages, with 60 to 100 percent takes.

During development of the leukemia, relative, and often moderate, absolute granulocytosis with final outburst of plasma cells into the blood was observed. At the same time increasing amounts of the pathological β-globulin fraction could be demonstrated by paper electrophoresis of the serum. At *post-mortem* examination, infiltrations of the ordinary localization seen in leukemia were found, particularly in the thymus, lung, liver, spleen, perirenal tissue, and lymph nodes.

The differentiation grade of the plasma cells was determined as grade II-III according to the criteria we used in earlier publications (*1*, *8*).

Serums from mice of 3 subsequent transplantation passages were used for the present immunoelectrophoretic investigations. These were the fourth, fifth, and sixth transfers, respectively. The leukemic mice were killed when moribund, and blood for the electrophoreses was collected from the axillary vessels.

Transplantation line 36.—This line developed in a 30-month-old male, which, at autopsy, showed enlargement of the spleen and lymph nodes, particularly the pancreatic and mesenteric nodes.

The spleen and lymph nodes were minced and injected subcutaneously into 6 (CBA $\times$ DBA/2)F_1 hybrid mice. The leukemia took in 5 mice; pronounced leukemic infiltrations were found in the liver, spleen, lungs, and lymph nodes. Paper electrophoresis showed that in 3 mice the leukemia was associated with the development of a γ-paraprotein fraction of M-type. Transplantation from 1 of these 3 mice resulted in transplantation line 36 y, in which the paraprotein fraction has remained unchanged in all mice examined through several passages. One of the 2 leukemias with no paraprotein fraction was also transplanted, thus starting transplantation line 36 x. Paper electrophoresis always failed to show abnormal serum-protein fractions in mice from several passages of line 36 x.

Except for the difference in electrophoretic findings, both lines behaved identically, *i.e.*, nearly 100 percent takes. The survival time, which in the first passage was about 11 months, shortened during the following transfers to less than 1 month after 8 to 10 passages.

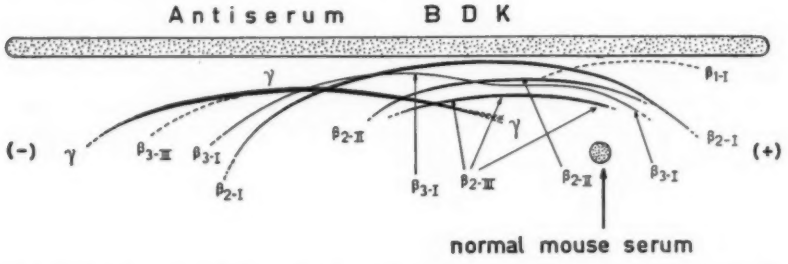
The blood changes were similar to those seen in transplantation line 20 except that the number of plasma cells found in the blood *sub finem* by far exceeded that seen in line 20.

Microscopic examination of the organs revealed heavy leukemic infiltration of the liver, spleen, perirenal tissue, and lymph nodes with plasma cells.

Serums from mice of the third and fifth passages of transplantation line 36 y (with paraprotein) and from the seventh and eleventh passages of transplantation line 36 x (without paraprotein) served as material for the present immunoelectrophoretic investigations. The mice were killed *in extremis*, and blood was procured by cutting the axillary vessels.

Immunoelectrophoresis.—The technique of immunoelectrophoresis was the same as previously described (*15*). Four different antiserums from rabbits against serum from normal (CBA × DBA/2)F_1 hybrid mice were used. These were called L-50, 229, 1-GR, and 2-HV, respectively. In the present paper the nomenclature of the various precipitation lines will be the same as that used in a previous publication on normal mouse serum (*15*), which described the existence of the following:

One γ-globulin, whose mobility extends far into the β-region.
Three β_2-globulins, $\beta_{2\text{-I}}$, $\beta_{2\text{-II}}$, and $\beta_{2\text{-III}}$; the latter is nearly always visible and has a doubly curved precipitation line.
Four β_2-globulins, $\beta_{2\text{-I}}$, $\beta_{2\text{-II}}$, $\beta_{2\text{-III}}$, and $\beta_{2\text{-IV}}$, of which only the first 3 are usually visible.
One β_1–globulin (see text-fig. 1).



TEXT-FIGURE 1.—Drawing of the β- and γ-regions of an immunoelectrophoresis of normal (CBA × DBA/2)F_1 hybrid mouse serum with labeling of the precipitation lines.

Results

Transplantation Line 20

Plasma-cell leukemia with β-paraprotein.—Plate 8 shows a confrontation between paper, agar, and immunoelectrophoresis of both a normal and a β-paraprotein-containing serum. The large β-paraprotein peak is strongly evident on paper and the conventional agar electrophoresis stained with Amidoschwarz 10 B. A reduction of the amount of protein normally present in the γ-region can also be noted. No significant changes appear to be present in the faster parts of the electrophoresis. Comparison between the normal and the pathological immunoelectrophoretic tracings is most instructive (plate 9, figs. 4 and 5).

The γ-bow is greatly deformed in its faster portions as it suddenly curves away from its normal direction and approaches the border of the antibody reservoir. Simultaneously, it gains considerably in intensity and sharpness. These facts are indicative of localized excess of antigen corresponding to the paraprotein of the conventional tracings.

The γ-line can be followed much more anodically than in the normal tracing, which is also explained by the local excess of antigen. The part of the γ-bow which is slower than its pathological portion is reduced in concentration.

Inspection of the other lines in the pathological region shows that $\beta_{2\text{-I}}$ and $\beta_{2\text{-II}}$, at first sight, do not seem to be modified; the former, however, is largely covered and masked by the huge paraprotein bow. On the other hand, the bow called $\beta_{2\text{-III}}$ cannot be identified at its normal place but, instead, a large arc of distinctly faster mobility appears in the β_1-region. This will temporarily be referred to as β_X.

All 3 passages of transplantation line 20 gave rise to immunoelectrophoretic patterns that were similar to the one described, which belonged to a mouse of the fourth passage called Tr. line 20_{4a}/7411. Therefore we will restrict further descriptions to serum obtained from this mouse.

Because the predominant abnormalities appeared to affect the γ-line and $\beta_{2\text{-III}}$, it seemed advisable to study this serum more extensively by means of the L-50 antiserum which contained powerful antibodies against both of these proteins.

In plate 9 several dilutions of pathological serum are compared with corresponding dilutions of normal mouse serum. The undiluted tracings (fig. 1, plate 9), as all following tracings developed with L-50, again show the abnormally increased γ-fraction of localized β-mobility. Similarly, the $\beta_{2\text{-III}}$-bow cannot be identified in its normal form, but a new bow of manifest β_1-mobility, previously referred to as β_X, can again be distinguished. Upon a 1 : 4 dilution of both normal and paraprotein serum with physiological saline (fig. 2, plate 9), the normal tracing of the β- and γ-regions is reduced to one distinct bow, $\beta_{2\text{-I}}$, because this is the most abundant protein in this area. The γ- and $\beta_{2\text{-III}}$-lines have faded into cloudy, weak streaks. The pathological serum, however, shows $\beta_{2\text{-I}}$ more clearly which suggests some increase of this protein; the really outstanding feature is the persistence of an extremely powerful, fast γ-bow corresponding to the paraprotein. The β_X is reduced to a faint remnant.

The 1:16 dilution of normal serum exhibits only a weak trace of $\beta_{2\text{-I}}$, which is more pronounced in the pathological serum (fig. 3, plate 9). The latter again shows a well-preserved, fast paraprotein γ-line.

At this point an effort was made to identify the real nature of the strange β_X bow, which apparently had no counterpart of similar mobility in normal serum. The following hypotheses were considered:

1) β_X could be a protein that is present only in trace amounts of normal serum. This would have justified its apparent absence in normal tracings and the presence of a corresponding antibody in our rabbit antiserums, since it is known that traces of antigen may give rise to appreciable antibody titers.

2) β_X could be some sort of Bence Jones protein; this is a low molecular-weight moiety, being synthesized by the tumor tissue and often, though not always, having antigenic groups in common with the paraprotein. Such proteins may have various electrophoretic mobilities in human myelomatous serums, sometimes even extending into the α-region. They often give rise to weak precipitation lines when studied immunoelectrophoretically with antiserums against normal human serum or against normal human γ-globulin (*17*).

3) β_X could be a modified form of $\beta_{2\text{-III}}$-mouse globulin. Several arguments seem to favor this hypothesis. It is remarkable that whenever this β_X-line appeared in the pathological serum the corresponding normal $\beta_{2\text{-III}}$-bow could not be visualized. Also, a confrontation of many β-paraprotein tracings with normal serum showed that the relative mobility of the β_X-bow was rather variable, and that all intermediate mobilities could be found between the extreme position in the β_1-region and the normal $\beta_{2\text{-III}}$-localization.

Thus, it appeared logical to study the behavior of the β_X-line in a mixture of normal and pathological serums. Plate 10, figure 1 shows a comparison between normal serum, β-paraprotein serum, and a mixture of equal parts of both, all developed with BDK antiserum. It can be seen that there is no intersection of two independent bows in the implicated region but that the β_X- and the normal $\beta_{2\text{-III}}$-bows are completely fused into each other, thereby establishing their total antigenic identity.

A confirmation was obtained from another experiment in which Tr. line 20_{4a}/7411 serum was developed with antiserum 229 (plate 10, fig. 2), which was known to reveal $\beta_{2\text{-III}}$ weakly in normal serums. A faint β_X-bow appeared according to our hypothesis that β_X was a $\beta_{2\text{-III}}$ of modified mobility.

Transplantation Line 36

As mentioned previously, transplantation line 36 had divided, in the first transfer passage, into 2 transplantation lines: line 36 y, associated with the development of γ-paraprotein of M-type, and line 36 x, which showed no abnormalities in paper electrophoresis.

Transplantation Line 36 y

Plasma-cell leukemia with γ-paraprotein of M-type.—Plate 11 confronts paper, agar, and immunoelectrophoresis of normal (CBA $\times$ DBA/2)F_1 hybrid mouse serum and γ-paraprotein-containing serum from mouse Nr. 7678 of the third transfer passage. The other mice from this transplantation line showed identical pictures.

On paper strip the abnormal fraction is clearly visible, but the conventionally stained agar tracing does not resolve it from the normal β-components because of the rapid diffusion taking place in this medium.

The immunoelectrophoretic pattern was developed with BDK antiserum (*15*). Two abnormalities are visible upon comparison with the parallel normal tracing. First, the γ-precipitation bow is deformed in its intermediate region where it suddenly deflects toward the antibody reservoir. Both the slower and faster part of the γ-bow are somewhat fainter than normal but situated, however, at their normal position. This indicates a very localized increase of γ-antigen in the implicated region, and translates the paraprotein peak of conventional paper electrophoresis.

The second peculiarity affects the $\beta_{2\text{-III}}$-protein whose precipitation line is somewhat intensified and has distinctly faster mobility than the corresponding normal line of standard pattern (*15*).

It could be shown that both this $\beta_{2\text{-III}}$-aberrant precipitation line as well as the corresponding $\beta_{2\text{-III}}$-bow in transplantation line 20 really represented modified $\beta_{2\text{-III}}$-globulin. This was demonstrated as described for transplantation line 20 by mixing normal and pathological serum in which both bows completely fused into each other.

Other lines, representing the $\beta_{2\text{-I}}$- and $\beta_{2\text{-II}}$-globulins, respectively, do not appear to be modified. The $\beta_{2\text{-I}}$-line was not clearly developed in the normal or pathological tracing and therefore had to be restudied with other antiserums.

As the most significant changes appeared to be associated with the γ- and the $\beta_{2\text{-III}}$-globulins, we decided to develop the paraprotein serum with the potent L-50 antiserum, which shows, particularly well, these protein lines.

Plate 12 illustrates the patterns obtained by the use of L-50 antiserum with 3 successive dilutions, 1:4, 1:8, and 1:16, respectively. Each pathological tracing is compared to a tracing of normal serum of similar dilution. Almost nothing of the normal γ-line remains visible with a 1:4 dilution (fig. 1, plate 12). On the contrary, the paraprotein serum shows a persistent visibility of the intermediate part of the same line, with a similar dilution; even when it is diluted 1:16, a cloudy streak still remains to attest its presence (fig. 3, plate 12). This clearly demonstrates the importance of the localized increase of γ-antigens.

As to $\beta_{2\text{-III}}$-globulin, it is obvious that the pathological precipitation line, as seen in a 1:4 dilution, has a faster than normal mobility and represents a rather considerably increased protein. Indeed, the normal, corresponding $\beta_{2\text{-III}}$ in such a dilution has given rise to no more than a faint precipitate.

A third abnormality has also become apparent by this dilution technique. The $\beta_{2\text{-I}}$-bow, which represents a protein that makes up most of the β-region of normal serum (*15*), is of exceptional sharpness and intensity even in the highest dilution, 1:16 (fig. 3, plate 12), where the corresponding normal precipitation lines is definitely fading.

At this point some doubt arose as to the real correspondence of this enlarged globulin fraction which we had somewhat confidently called "$\beta_{2\text{-I}}$," with the normal, $\beta_{2\text{-I}}$. Indeed, as can be seen from all illustrations, the $\beta_{2\text{-I}}$-line and the pathological $\beta_{2\text{-III}}$-line join each other in their faster part. This could very well be accidental overlapping but some antigenic connection could not be excluded without further investigation. As we disposed of a preparation of mouse $\beta_{2\text{-I}}$-globulin, it was possible to specifically eliminate the corresponding antibody from antiserum BDK and to reinvestigate the M-paraprotein with such absorbed antiserum.

The result is shown in plate 13, figure 1. The absorbed antiserum was used to fill the central antibody reservoir, while nonabsorbed BDK was used for the lateral reservoirs. Both diluted (1:4) and undiluted paraprotein serum was submitted to the immunoelectrophoresis. Clearly, the absorbed antiserum failed to develop the $\beta_{2\text{-I}}$-protein without affecting the $\beta_{2\text{-III}}$-protein, thus justifying our assumption that both proteins were

antigenically independent and corresponded to their similarly labeled counterparts in normal serum.

Transplantation Line 36 x

Plasma-cell leukemia without paraprotein.—Plate 14 confronts paper, agar, and immunoelectrophoresis of normal (CBA × DBA/2)F_1 hybrid mouse serum and serum without paraprotein from mouse Nr. 7828 of the seventh transfer passage. The other mice from this transplantation line showed identical results. No significant abnormality can be seen on either paper or agar electrophoresis.

No paraprotein-like enlargement of the γ-line is visible on the immunoelectrophoretic tracing. Quite the contrary, the whole of the γ-bow appears much fainter than with normal serum. Yet the area corresponding to that part of the γ-bow where the paraprotein of transplantation line 36 y is localized still contains the greatest amount of the γ-protein, as is seen from a relative condensation of the protein bow in this localization.

As in transplantation line 36 y (with paraprotein) the same abnormally fast and intensified $\beta_{2\text{-III}}$-line may be seen. Remnants of it persist as a cloud with a dilution of 1:4 (plate 13, fig. 2).

In contrast to the serum with paraprotein, no abnormal increase of the $\beta_{2\text{-I}}$-globulin appears to be present, as can be deduced both from diluted (plate 13, fig. 2) and undiluted serum tracings (plate 14, fig. 4).

Conclusion and Discussion

The immunoelectrophoretic studies of the β-paraprotein of plasma-cell leukemia transplantation line 20 have revealed an increased intensity and a deformation of the γ-bow in its faster portions; these findings are due to a considerable increase of the γ-protein precipitated in this localization. Furthermore, the $\beta_{2\text{-III}}$-line showed a distinctly faster mobility and a greater intensity than in normal serum. The $\beta_{2\text{-I}}$-bow was also increased to some extent.

An obvious conclusion of these findings is that the name *beta type of plasma-cell leukemia* initially bestowed upon this paraprotein-developing leukemia can only be taken as descriptive of the electrophoretic mobility of the abnormal protein fraction. If an immunochemical viewpoint is adopted, however, it should more correctly be called a *gamma type of plasma-cell leukemia of beta mobility.*

This point is of interest for several reasons. A similar situation has been repeatedly described (*17–19*) in human myeloma disease. Some human myeloma paraproteins of β-mobility were shown to be quantitatively and qualitatively abnormal forms of a human β-protein, which Burtin and coworkers (*20*) called β_{2A} and which was isolated and described by two of us (*21*). Many electrophoretically similar, human β-paraproteins, however, appeared to be derived from the faster part of normal, human γ-globulin. Such relationships already were vaguely suggested by earlier immunochemical work (*22*, *23*), but it is obvious that immunoelectrophoresis was really the method for their precise classification.

The present studies of the subline of plasma-cell-leukemia transplantation line 36 y, which was associated with the development of γ-paraprotein of M-mobility, revealed pathological changes that in many respects appear to be homologous to those described for the β-type. Again the same three proteins, γ-, $\beta_{2\text{-III}}$-, and $\beta_{2\text{-I}}$- are affected. The γ-line shows the characteristic paraprotein deflection corresponding to the M-area of ordinary electrophoresis. There is both an increase and a velocity change of $\beta_{2\text{-III}}$-globulin, which extends too far anodically; an important increase of the concentration of $\beta_{2\text{-I}}$-globulin completes the picture.

Consequently, except for the precise location of the paraprotein abnormality of the γ-globulin, the qualitative biochemical changes for the two paraprotein types may be superimposed.

From a quantitative point of view, however, the immunoelectrophoresis shows some striking differences between β- and γ-paraprotein-developing plasma-cell leukemias. The abnormal portion of the γ-antigen is quantitatively much more important in the β- than in the M-type, a fact already apparent from paper electrophoresis. Conversely, the relative increase in $\beta_{2\text{-I}}$-globulin behaves in the opposite way; it is easily overlooked in the β-type, while it is quite striking for the γ-type. These discrepancies are best revealed by using antiserum L-50.

In the transplantation line 36 x, which showed no paper-electrophoretic changes, immunoelectrophoresis revealed a γ-bow of normal position but definitely much fainter than in normal serum. The abnormally fast and intensified $\beta_{2\text{-III}}$-line, found in transplantation line 36 y with γ-paraprotein and in the β-paraprotein-containing serum of line 20 as well, was also present in this serum without the γ-paraprotein, whereas no increase in $\beta_{2\text{-I}}$ could be noticed in contrast to the findings in the abnormal serums just mentioned.

Another point deserves some attention. Both for the β- and the γ-type plasma-cell leukemia, it may be noted that the electrophoretic portions of the γ-globulin which are not involved in the paraprotein transformation are of reduced concentration. This applies also to the whole γ-bow in the serum from mice of transplantation line 36 x without paraprotein. Similar findings are often reported in the serums of human beings with myelomas and offer speculation as to what really happens to normal globulin-forming tissue when a plasma-cell neoplasia invades the organism. We do not know to what extent the reported increase of $\beta_{2\text{-I}}$ can be correlated to the leukemic process.

We also do not know the significance of the changes we observed in the behavior of the $\beta_{2\text{-III}}$-globulin, consisting of an increased mobility and a distinctly larger concentration when compared to normal standards. However, it may be mentioned that these findings are reminiscent of the changes in $\beta_{2\text{A}}$-globulin described by Grabar and coworkers (*17*), which are often associated with various forms of human myeloma disease. Work on the establishment of homology between human and mouse proteins, as well as on the pathology of $\beta_{2\text{-III}}$-globulin in other diseases of mice, is currently in progress.

Comparing the results of paper and immunoelectrophoresis it will be seen that paper electrophoresis was unable to reveal the increase of the $\beta_{2\text{-III}}$-lines found in all 3 lines of leukemia, as well as the presence or absence of the increase of $\beta_{2\text{-I}}$. And last but not least, paper electrophoresis was unable to demonstrate that the paraprotein of transplantation line 20 was not a β-paraprotein but a γ-paraprotein of β-mobility.

Thus it is evident that the application of the immunoelectrophoretic technique has brought important additional contributions to the elucidation of the serological changes observed in paper electrophoresis on serums from mice carrying plasma-cell leukemias.

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PLATE 8

Comparison between normal serum and serum from transplantation line 20 with β-paraprotein.

FIGURE 1.—Conventional paper-strip electrophoresis of normal serum.

FIGURE 2.—Conventional paper-strip electrophoresis of pathological serum.

FIGURE 3.—Conventional agar electrophoresis of pathological serum.

FIGURE 4.—Immunoelectrophoresis of pathological serum, developed with antiserum BDK.

FIGURE 5.—Immunoelectrophoresis of normal serum, developed with antiserum BDK.

FIGURE 6.—Scanning diagram of paper-strip electrophoresis of normal and pathological serum.

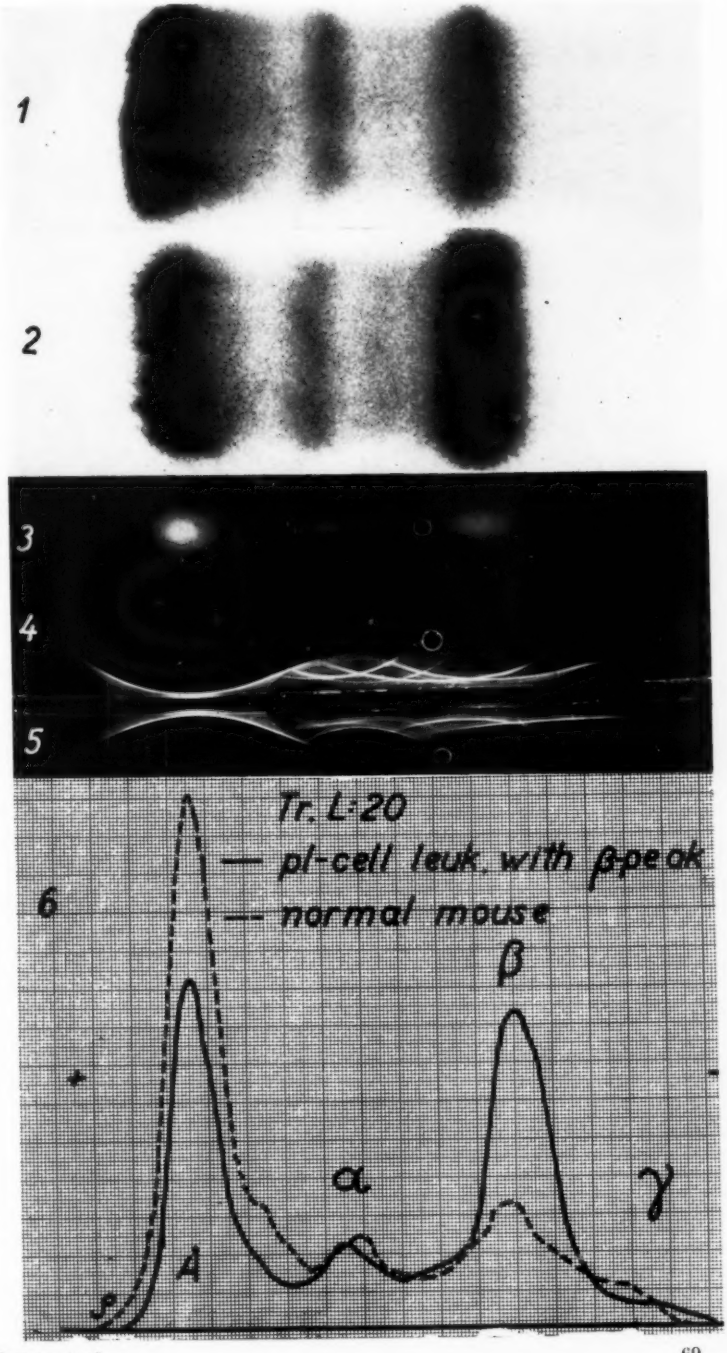

1
2
3
4
5
6
Tr. L:20
— pl-cell leuk. with β peak
-- normal mouse
β
α
γ
A
+
-

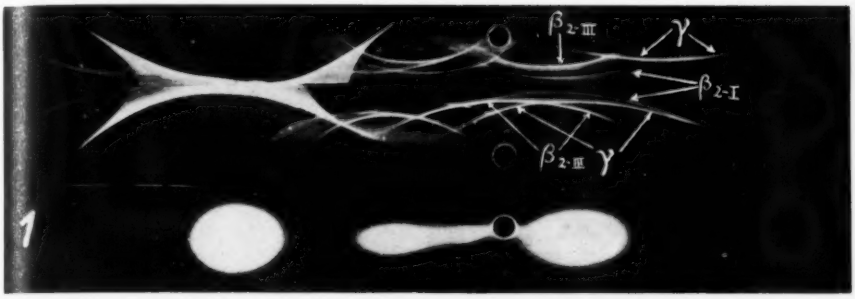
PLATE 9

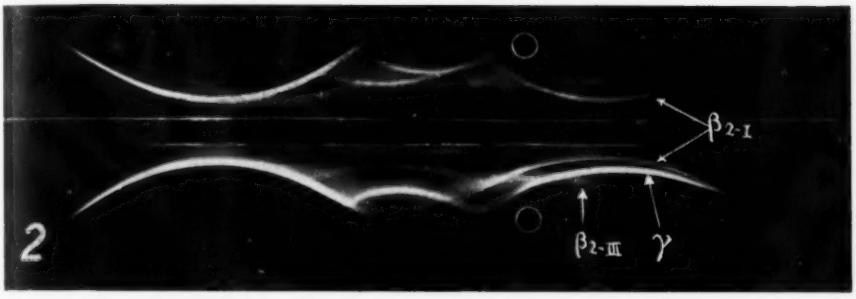
Immunoelectrophoresis of normal and β-paraprotein-containing serum, undiluted and diluted 1 : 4 and 1 : 16, developed with antiserum L-50.

FIGURE 1.—*Upper* part: Immunoelectrophoresis of normal serum with labeling of the significant bows.
Central part: Immunoelectrophoresis of pathological serum with labeling of the significant bows.
Lower part: A conventional agar electrophoresis of pathological serum.

FIGURE 2.—*Upper* part: Immunoelectrophoresis of normal serum, diluted 1 : 4.
Lower part: Immunoelectrophoresis of pathological serum, diluted 1 : 4.

FIGURE 3.—*Upper* part: Immunoelectrophoresis of normal serum, diluted 1 : 16.
Lower part: Immunoelectrophoresis of pathological serum, diluted 1 : 16.


$\beta_{2\text{-III}}$
γ
$\beta_{2\text{-I}}$
$\beta_{2\text{-III}}$
γ
1


$\beta_{2\text{-I}}$
$\beta_{2\text{-III}}$
γ
2

$\beta_{2\text{-I}}$
γ
3

PLATE 10

FIGURE 1.—Confrontation of immunoelectrophoresis of normal serum, a mixture of equal parts of normal and β-paraprotein-containing serum, and β-paraprotein-containing serum, developed with antiserum BDK.
a) Immunoelectrophoresis of normal serum.
b) Immunoelectrophoresis of equal parts of normal and pathological serum.
c) Immunoelectrophoresis of pathological serum.

FIGURE 2.—Immunoelectrophoresis of β-paraprotein-containing serum, undiluted, and diluted 1 : 4, developed with antiserum 229.
a) Immunoelectrophoresis of pathological serum, undiluted.
b) Immunoelectrophoresis of pathological serum, diluted 1 : 4.

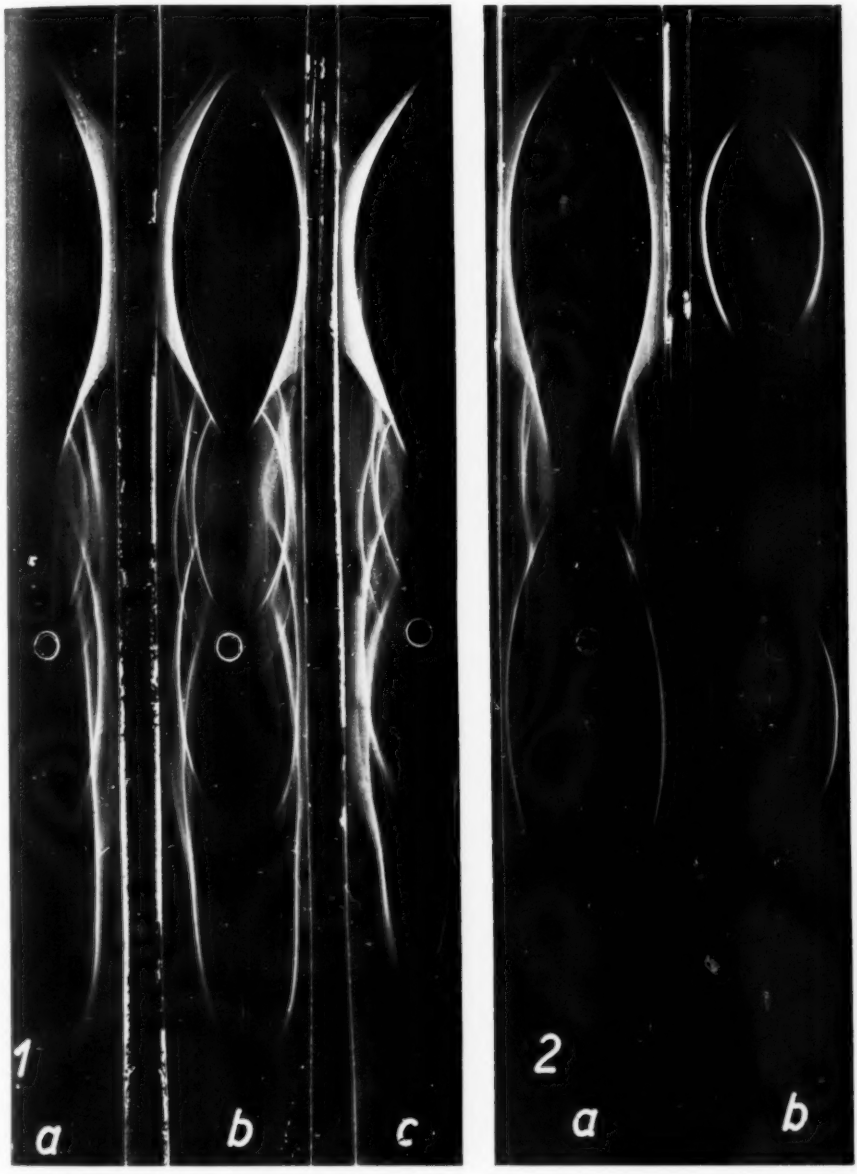

1
a
b
c
2
a
b

PLATE 11

Comparison between normal serum and serum from transplantation line 36 y (with γ-paraprotein).

FIGURE 1.—Conventional paper-strip electrophoresis of normal serum.

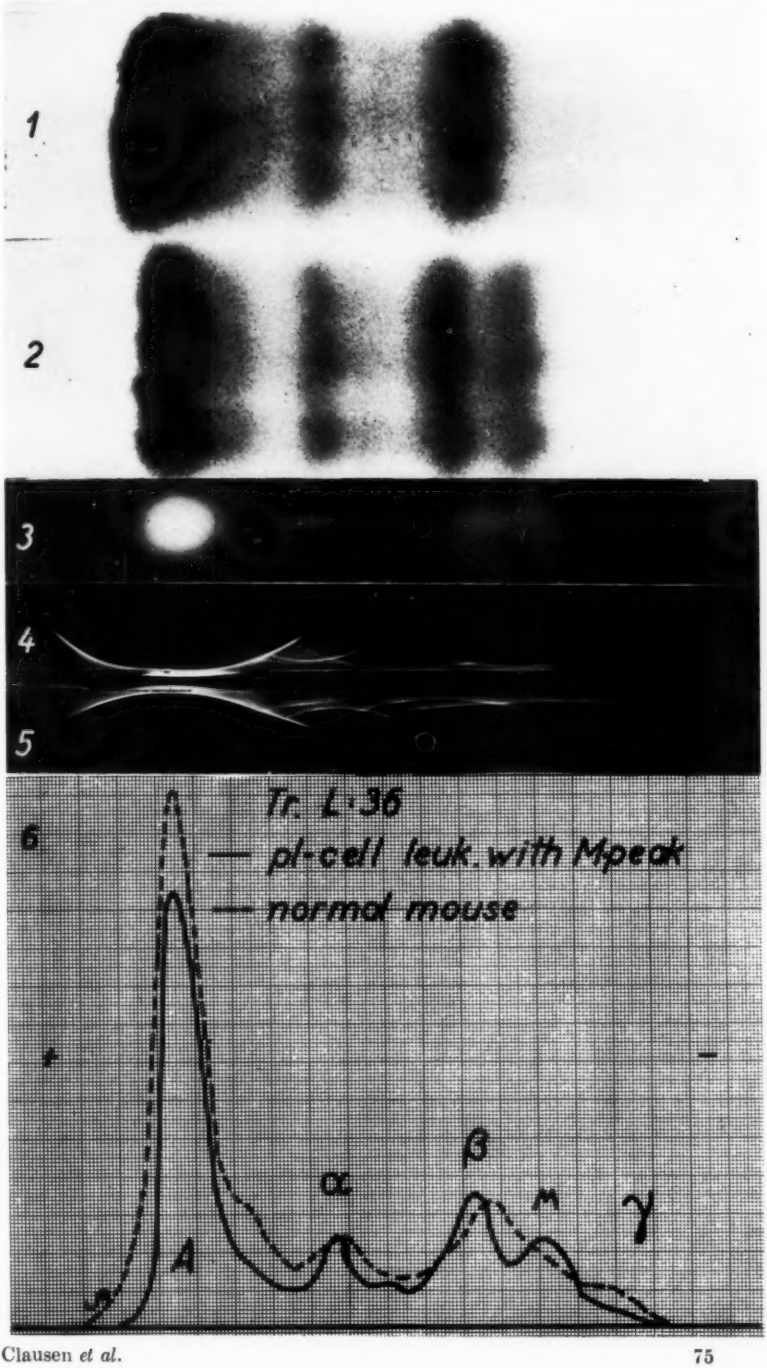
FIGURE 2.—Conventional paper-strip electrophoresis of pathological serum.

FIGURE 3.—Conventional agar electrophoresis of pathological serum.

FIGURE 4.—Immunoelectrophoresis of pathological serum, developed with antiserum BDK.

FIGURE 5.—Immunoelectrophoresis of normal serum, developed with antiserum BDK.

FIGURE 6.—Scanning diagram of paper-strip electrophoresis of normal and pathological serum.



488412—59——10

PLATE 12

Immunoelectrophoresis of normal serum and serum from transplantation line 36 y (with γ-paraprotein) diluted 1 : 4, 1 : 8, and 1 : 16 and developed with antiserum L-50.

FIGURE 1.—*Upper* part: Immunoelectrophoresis of normal serum, diluted 1 : 4.
Lower part: Immunoelectrophoresis of pathological serum, diluted 1 : 4.

FIGURE 2.—*Upper* part: Immunoelectrophoresis of normal serum, diluted 1 : 8.
Lower part: Immunoelectrophoresis of pathological serum, diluted 1 : 8.

FIGURE 3.—*Upper* part: Immunoelectrophoresis of normal serum, diluted 1 : 16.
Lower part: Immunoelectrophoresis of pathological serum, diluted 1 : 16.

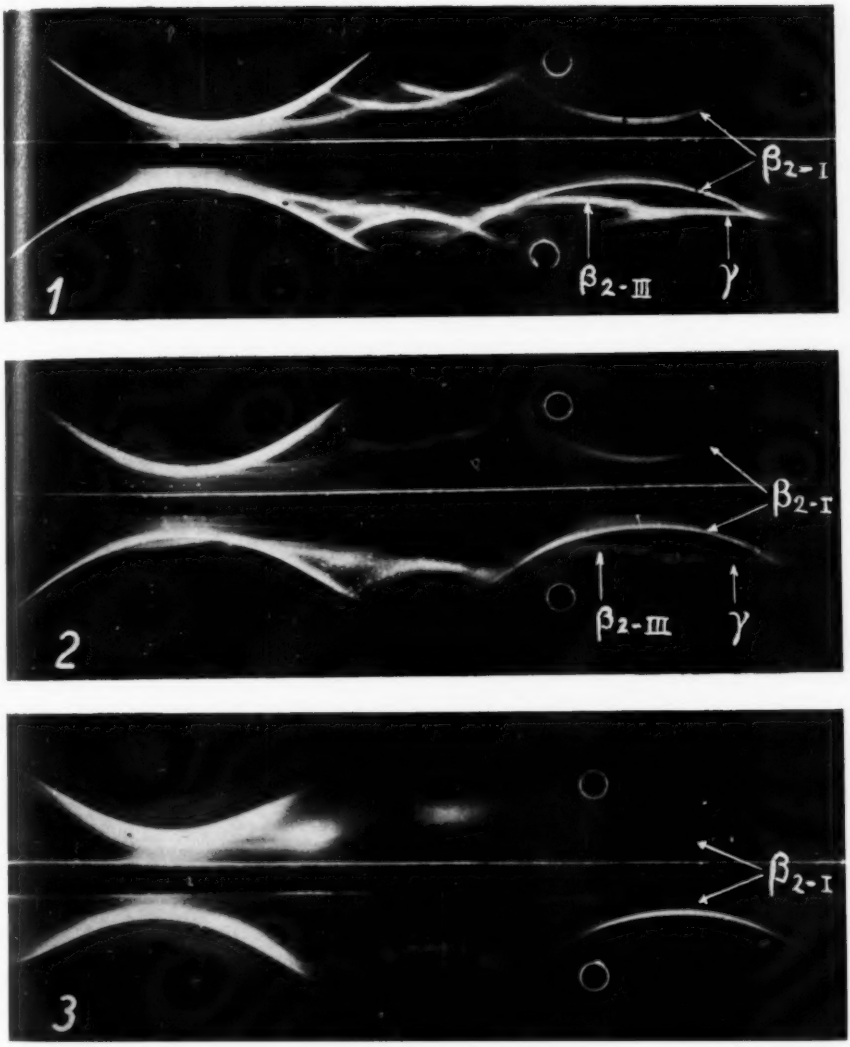

β2-I
β2-III
γ
1
β2-I
β2-III
γ
2
β2-I
3

PLATE 13

FIGURE 1.—Immunoelectrophoresis of serum from transplantation line 36 y (with γ-paraprotein), diluted 1 : 4 and undiluted, developed with antiserum BDK, nonabsorbed and absorbed with $\beta_{2\text{-}1}$-globulin.

a) Immunoelectrophoresis of pathological serum, diluted 1:4, and developed with antiserum BDK, nonabsorbed (lateral reservoir) and with antiserum BDK absorbed with $\beta_{2\text{-}1}$-globulin (central reservoir).

b) Immunoelectrophoresis of pathological serum, undiluted and developed with antiserum BDK, nonabsorbed (lateral reservoir) and with antiserum BDK, absorbed with $\beta_{2\text{-}1}$-globulin (central reservoir).

FIGURE 2.—Immunoelectrophoresis of normal serum and serum from transplantation line 36 x (without paraprotein), developed with antiserum L-50.

a) Immunoelectrophoresis of normal serum, diluted 1:4.

b) Immunoelectrophoresis of pathological serum, diluted 1:4.

1a
b
2a
b

PLATE 14

Comparison between normal serum and serum from transplantation line 36 x (without paraprotein).

FIGURE 1.—Conventional paper-strip electrophoresis of normal serum.

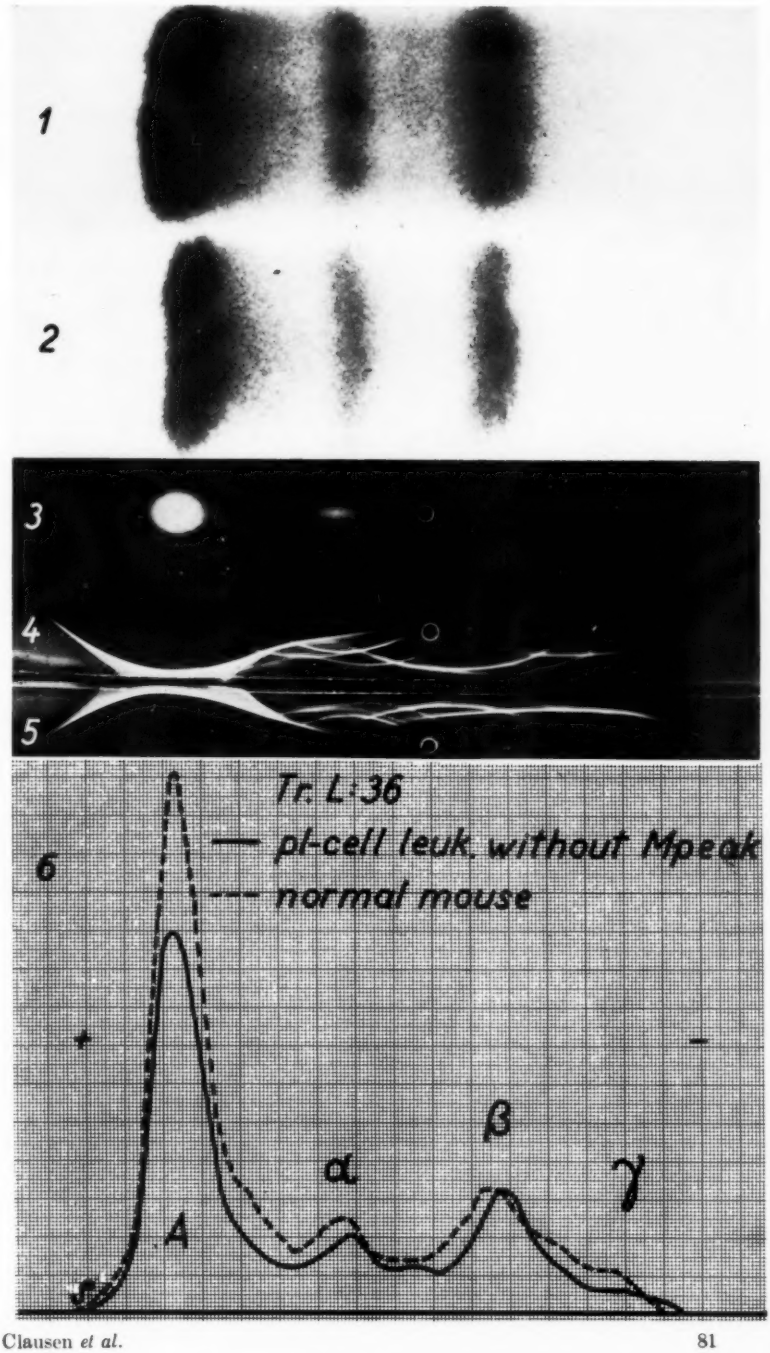
FIGURE 2.—Conventional paper-strip electrophoresis of pathological serum.

FIGURE 3.—Conventional agar electrophoresis of pathological serum.

FIGURE 4.—Immunoelectrophoresis of pathological serum, developed with antiserum L-50.

FIGURE 5.—Immunoelectrophoresis of normal serum, developed with antiserum L-50.

FIGURE 6.—Scanning diagram of paper-strip electrophoresis of normal and pathological serum.


1
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Tr. L:36
— pl-cell leuk. without M peak
--- normal mouse
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Pathogenicity of a Viral Strain (RPL12) Causing Avian Visceral Lymphomatosis and Related Neoplasms. I. Nature of the Lesions[1]

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Summary

Morphologic studies of lesions in chickens of varied breeding experimentally exposed to strain RPL12 virus reveal that four principal multicentric neoplastic entities are elicited by this strain. Two of these, erythroblastosis and visceral lymphomatosis, are highly malignant; one, osteopetrosis, eventually subsides; and one, hemangiomatosis, is unquestionably benign. The pathogenesis of erythroblastosis is compared and contrasted to that of visceral lymphomatosis. It is concluded that the latter is an extravascular neoplasia arising both intramedullarly and extramedullarly in a multicentric manner. Occasionally, a leukemic or leukemoid state is present; frequently, a secondary erythroid hyperplasia in the marrow is associated with this disease. Comparative studies on normal lymphoid foci, transplantable lymphoid tumors, field cases of the disease, and non-neoplastic pathologic proliferations suggest that the cell type in visceral lymphomatosis is a highly undifferentiated element of the lymphoid series. The experimental disease is equivalent to field cases of visceral lymphomatosis and to the entity described as lymphoid leukosis. The lesions in osteopetrosis and hemangiomatosis are briefly described and illustrated. Within each of these types of disease, the character of the lesions is not affected by such variables in the host as age, sex, and genetic background, or by the conditions of exposure to the virus. Nor is there any variation in this respect provided by other sources of serially propagated or freshly isolated virus.—**J. Nat. Cancer Inst. 22: 83–101, 1959.**

In previous studies (*1*, *2*) on the pathogenicity and other host relations of strain RPL12 virus, mention was made of the principal disease manifestations of the agent: extravascular lymphomatosis, intravascular lymphomatosis, and osteopetrosis. Extensive comparative histopathologic studies made during the past year reveal that the entity referred to as intravascular lymphomatosis is in reality erythroblastosis.

The present report describes the characteristic lesions in each of these types of disease and their differential features, and attempts to analyze the sequence of tissue changes. The second paper (*3*) will consider in

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488412—59——11

detail the various factors which influence the type and frequency of the diseases caused by this strain.

Materials and Methods

The observations pertain primarily to routine *post-mortem* examination of birds in experiments described in detail in the second paper (*3*). Most of the chickens were single-comb White Leghorns of inbred line 15, which is susceptible to visceral lymphomatosis and which has been maintained in isolation for many years. However, some birds from other inbred lines with different susceptibility and some of other breeds from field flocks were used. Disease in these birds arose either naturally or as a result of experimentally controlled exposure to RPL12 cell-free material (passages 15 to 17). The controlled exposure was accomplished by inoculation or by artificial contact. This tumor strain was originally isolated and described by Olson (*4*), who propagated it as a cellular transplant through 138 passages. In our laboratory, 85 additional serial transplant passages were made prior to the series of cell-free passages.

In order to determine the specificity of the findings, comparative studies were made of lesions caused by other isolated or propagated cell-free material, filtered tissue-culture propagated material, and transplanted tumor. For special experiments on pathogenesis, the birds were killed at definite intervals of time after exposure. Sections of tissues were fixed in either Zenker-acetic or Zenker-formol and stained with hematoxylin-triosin; blood and tissue imprint smears were stained with May-Grünwald-Giemsa stain. Details on the preparation of cell-free and cell-suspension inoculums, as well as on the management of the experimental birds, have been published (*2*, *5*, *6*).

Rather than tabulate the various experimental conditions in detail, these will be mentioned wherever relevant.

Results and Discussion

In addition to the manifestations previously mentioned, a fourth entity, hemangiomatosis, which was reported for other propagated strains (*7*), is associated also with certain passage series of RPL12 (*3*). It is worthy of emphasis at this point that RPL12 virus does not elicit neural and ocular lymphomatosis, myelocytomas, or any epithelial tumors; fibrosarcomas and myxosarcomas (*1*) are very infrequent. This report will therefore be confined to the four principal pathologic manifestations of strain RPL12: erythroblastosis, visceral lymphomatosis, osteopetrosis, and hemangiomatosis.

Erythroblastosis

This highly malignant neoplasm of the bone marrow is only rarely diagnosed in field flocks, yet its occurrence among birds inoculated with cell-free material from various sources is widespread. It is a cardinal feature not only of RPL12 but also of other propagated virus strains,

such as RPL16, 18, 19, 20, 21, 23 (*3*), and some strains maintained at other research centers (*8*). Interestingly, none of the RPL strains was originally isolated from birds with erythroblastosis—the donor birds invariably had visceral lymphomatosis only—yet erythroblastosis became manifest in the recipients during the first few passages (*3*). The disease is seldom acquired through contact exposure, although this statement is not meant to reflect on the contact transmissibility of the agent itself.

In birds with erythroblastosis there are no significant differences in the nature of the lesions that occur after inoculation with any of the strains listed or with such "natural" sources of virus as: 15-day embryo-liver extract from eggs laid by normal-appearing hens, filtered oral washings or fecal extracts of lymphomatous birds (natural infection), and filtered extracts of incubator debris. Nor are differences observed among the few available cases where the disease arose through artificial contact exposure to a flock naturally infected with visceral lymphomatosis. Age, sex, breed, or genetic constitution likewise appear to have no influence on the character of the lesions. Yet, the incidence of the disease and, to a certain degree, its incubation period are affected by such factors as origin of the virus, dose, age at inoculation (*3*), and genetic susceptibility of the host (*9*).

The pathogenesis of erythroblastosis has been well described recently by Pontén and Thorell (*10*), who worked with a strain isolated by Engelbreth-Holm (probably strain R). Except for the time relations, the sequence of tissue changes in this disease as produced by RPL12 is identical with that described by these authors. It is noteworthy that the neoplastic cell—the proerythroblast as designated by Ferrata (*11*) and Bessis (*12*)—arises in the bone marrow only; also a leukemic state becomes evident only after the marrow sinusoids present 2 or 3 continuous concentric layers of neoplastic proerythroblasts, which are uniform in appearance. Pontén and Thorell's observations that these cells are not present extramedullarly before the existence of a leukemic state are also confirmed. Thus, the strictly intravascular nature and the exclusive bone marrow origin of erythroblastosis sharply differentiates the character of this disease from that of other members of the avian leukosis complex. That erythrogenesis is a strictly intravascular process even in normal adult fowl has been pointed out by Denys (*13*), Bizzozero (*14*), Doan (*15*, *16*), Sabin (*17*), and Furth (*18*). The malignancy of erythroblastosis has been recognized by Campbell (*19*), and, with respect to the neoplastic-cell type, Darcel (*20*) is of the opinion that it is more primitive than the erythroblast (presumably in Ferrata's classification). Under these assumptions, the suggestion of the term "erythroblastoma" (*21*) for a liver lesion in a bird intramuscularly inoculated with transplantable lymphoid tumor ought to be taken with reservation, particularly since the authors fail to describe the appearance of bone marrow or liver sections. Imprint smears rarely reflect the proportions of the various cell types found in tissue sections and they may, even after repeated blotting, give an impression of sinusoidal blood only.

With respect to the incubation period, RPL12 differs from the strain studied by Pontén and Thorell in both the length and the variability of this period (*3*). This difference can be at least partially accounted for by the difference in virus dose used. As to the course of RPL12-induced erythroblastosis, 5 to 10 days elapse between the appearance of peripheral-blood changes and death. From experiments on pathogenesis it has been estimated that microscopic manifestation of erythroblastic neoplasia is evident in the bone marrow 2 or 3 days before the appearance of neoplastic cells in the circulating blood.

The grossly observed lesions in erythroblastosis reflect the microscopic changes: a slight swelling of the liver and spleen at the time leukostasis in the parenchymatous organs reaches the stage depicted in figure 4, and a terminal, acute, generalized passive hyperemia, often accompanied by a gelatinous transudate, in the peritoneal cavity (fig. 1).

The fact that no gross or microscopic evidence of pulmonary edema or hepatic degeneration is seen suggests that death is sudden and probably due to circulatory and respiratory failure.

Visceral Lymphomatosis

This disease is the second principal manifestation of RPL12 and, except for differences in incidence, length of incubation period, and course, it appears to be similar to visceral lymphomatosis elicited by every other strain propagated in this laboratory. Differences in the host, such as genetic resistance, sex, age at exposure, and age at death, do not appear to influence the character of the lesions. Neither do conditions of exposure, such as method, route (if by inoculation), dose, and whether the agent has been repeatedly propagated in serial passage or freshly isolated from a variety of sources. As with erythroblastosis, certain conditions of exposure have a marked effect on the general incidence and time-response relations (*3*).

Unlike erythroblastosis, this neoplasm arises both intramedullarly and extramedullarly in a multicentric manner. The liver, spleen, and bone marrow are almost always involved—the kidneys only slightly less frequently. Tumorous lesions in the gonads, particularly the ovary, are next in order of frequency, and somewhat less commonly the bursa of Fabricius is involved. Occasionally, lesions are seen in the intestinal wall, myocardium, voluntary musculature, subcutaneous tissue, and the lung. The central and peripheral nervous system and, oddly, the thymus are only very rarely tumorous. The detailed frequency of the various organs affected has been published (*6*).

Birds suffering from the disease are apt to be debilitated and in poor general condition. The enlarged liver can usually be palpated in the live bird as a fairly massive structure projecting beyond the costal arch and partly or fully covering the gizzard.

The structure of the lesions is somewhat variable, but no association of any particular pattern can be made with any of the various factors studied or even with the degree of cell differentiation. The liver is practically

always enlarged to a much greater extent than in erythroblastosis. Usually the neoplasia in this organ is diffuse or diffuse and focal (fig. 2). Miliary involvement is somewhat less common and the nodular type still more infrequent. Regardless of the distribution of the liver lesions, the spleen almost always shows a focal or coalescing focal neoplasia. As with the liver, the spleen is usually very large. Tumors in other tissues vary between the focal nodular and diffuse types.

Microscopically, most lesions are multicentric in origin and of a focal distribution; even in tissues grossly observed to be diffusely involved, the microscopic pattern is usually one of coalescing tumorous foci, which appear to have arisen simultaneously yet independently of each other. The growth of these foci is by invasion and destruction of the normal tissue rather than by infiltration (figs. 5 and 8).

The predominant cell type in tumors obtained by artificial exposure (inoculation or contact) to RPL12, as well as to other propagated or freshly isolated virus, and in all the different types of hosts studied is uniform and identical. No variation is provided by different tissues examined, stage of the disease process, or any other variable factors studied. Apparently, the only consideration of importance in this respect is whether the exposure is experimental or under field conditions. This will be discussed in more detail later.

With artificial exposure methods the neoplastic cell is a very immature form which, on the basis of comparative studies of normal birds and diseased birds from field flocks, has been placed in the lymphocytic developmental series. Various names have been proposed for this cell type: lymphoid cell, hemocytoblast, reticulolymphocyte, and prolymphoblast. While it is not within our scope to debate the merits of each of these terms, we shall, nevertheless, indicate the salient features of this cell.

In tissue sections (fig. 8) the neoplastic cells grow in massive aggregations, causing a loss of individual cell outline. The cytoplasm is somewhat less basophilic than that of proerythroblasts, and there is also a slightly larger N/C ratio than in the latter cells. The nuclei appear vesicular, because of margination and clumping of the chromatin into a few massive aggregates, and there are conspicuous acidophilic nucleoli. Sometimes the nucleoli are so prominent that they simulate the typical viral intranuclear inclusions of other diseases. The whole appearance of the cell suggests a highly undifferentiated form which bears a superficial resemblance to the neoplastic cell type of erythroblastosis—the proerythroblast. The most important differential feature that distinguishes this cell from the proerythroblast, besides its location and the general character of the lesion, is its great variability. Contrary to the very young erythroblasts, which appear very much alike (fig. 7), in visceral lymphomatosis there is considerable variation in the size and shape of the neoplastic cells. There is, however, no variability as far as maturation is concerned; all cells are at the same primitive developmental stage.

In tissue-impression smears of affected organs (fig. 9) a greater variability again is apparent in the appearance of the cells in comparison with

the proerythroblasts. Other differences include an ovoid rather than spherical-shaped nucleus and a finer textural quality in the chromatin network than that in immature erythroid cells. For this reason, the nucleoli so readily visible in sectioned material become obscured in smear preparations by the overlying chromatin. With ordinary illumination it is difficult to discern the nucleoli, but they become more readily visible when the light is passed through a combination of Wratten filters B and E.

Unlike the proerythroblast, this immature cell arises extravascularly. It can be found in the perivascular lymphoid foci of normal chickens and is generally assumed to be an undifferentiated (stem) cell of the lymphocytic series (*22*).

Comparative studies on the sequence of changes that arise following local inoculation of tumor-cell suspensions (RPL12 and RPL16) reveal that, at least under our present conditions of preparation with routine fixatives and stains, the cell type of the primary tumor at the site of inoculation is similar to that in tumors elicited by cell-free material. Working with cell transplants of tumor from which RPL12 had originated, Olson (*4*) refers to the tumor cells as a "neoplastic lymphoid" type.

A conception of the developmental relations of the neoplastic cells can be formed through a study of field cases of visceral lymphomatosis, which range continuously from the benign to the highly malignant. It is significant to note, however, that variations in malignancy occur among affected birds or even among different infected flocks, rather than among various neoplastic tissues or parts of a given neoplasm within a single host.

From the standpoint of equating the disease elicited by artificial exposure to that arising under field conditions, the naturally infected birds with highly anaplastic tumor cells (fig. 15) are most useful for comparative studies. The gross and microscopic appearance of their lesions is indistinguishable from that of birds infected with RPL12. Moreover, artificial exposure (whether by contact or inoculation) to other propagated strains or to virus in its "natural" form, such as cell-free oral washings, fecal extract, and incubator debris, results in tumors of an identical type. Many of the individual donor trials were made with birds that appeared free of lesions, yet in all the recipient birds examined the tumor cells were of this anaplastic type.

The naturally occurring tumors, in which considerable variability in the differentiation of the neoplastic cells is the paramount feature, have been considered an intermediate grade of malignancy. This type of lesion perhaps best serves to support the contention that the highly undifferentiated neoplastic cells properly belong to the lymphocytic developmental series; as far as can be determined, the immature cells observed in these tumors are indistinguishable from those described earlier, and the most differentiated ones appear to be mature lymphocytes (fig. 16).

The presence of chronic inflammation and fibrosis suggests that these tumors are of a slower-growing type.

At the lower end of the malignancy scale it is sometimes difficult to

distinguish accumulations of highly differentiated lymphoid cells from normal lymphoid foci or from nonspecific lesions that represent merely a state of heightened tissue reactivity to a variety of stimuli. Large lesions, in which definite tissue destruction had taken place, could be thought of as neoplastic and therefore properly fulfilling the defining criteria of visceral lymphomatosis (fig. 17); for those of a less dramatic character, there is no easy solution to prevent ambiguities.

In contrast to the view expressed by many (*23–32*), some authors (*33–37*) have even gone so far as to suggest that *all* lymphoid areas in the fowl are abnormal. This interesting conclusion, however, is mainly based on an ontogenic definition of normality, a questionable interpretation of tissue destruction, and a regression analysis between dependent variables (*34*).

It has long been recognized that a leukemic state can be present in visceral lymphomatosis. In a small proportion of the more malignant field cases, as well as in some of the RPL12 inoculated chickens, a leukemic state was observed. The significance of this finding is not wholly clear since there are no other differences from comparative aleukemic cases. The transmissibility of these tumors does not appear to be different from that of ordinary aleukemic ones, nor is there any assurance of perpetuating the leukemic state on further serial passage. While these infrequent cases may represent merely an accidental erosion of a vessel wall by the neoplasm (leukemoid state), it is also likely that a true lymphatic leukemia exists in the chicken. The fact that lymphoid cells arise extravascularly does not militate against this possibility, since the same is true of the obviously leukemic granuloblastosis.

Considerably more important is the fact that erythroid hyperplasia, which often occurs in this disease, may hinder the making of an *ante-mortem* differential diagnosis based on peripheral blood changes. Over half the experimental cases (fig. 6) and even some of the field cases of intermediate malignancy show an increased number of erythroblastic cells within the blood sinusoids of the marrow when this is extensively involved with extravascular lymphoid neoplasia. This proliferation is reflected in the peripheral blood where immature erythroid and thromboid cells at all stages of maturity are seen. Their number in the blood compares with that seen in the middle phases of erythroblastosis. Yet, this erythroid proliferation presents no evidence whatsoever of neoplasia: There is no expansion of the marrow sinusoids, no destruction of adjacent extravascular tissue, and the process is not progressive. Furthermore, the erythroid cells manifest gradual transitions in differentiation, a feature notably absent in erythroblastosis. Again, no abnormal erythroid activity is present extramedullarly. This proliferation appears to have all the characteristics of a compensatory hyperplasia on the part of a very sensitive cell system. Throughout this study, not a single case was observed in which erythroblastosis and visceral lymphomatosis were jointly present.

Photomicrographs of such non-neoplastic reactions as neural lymphomatosis (fig. 19) and nonspecific focal granuloma (fig. 20) are also included;

both are the result of chronic inflammatory change in which the reticuloendothelial system is involved. Since there are lymphocytes in all stages of development in both types of lesions and since the most immature forms are identical with the neoplastic cell in visceral lymphomatosis, the contention that this latter cell is committed to a lymphocytic line of development receives further indirect support.

Granuloblastosis (myeloblastosis, myeloid leukosis) is another neoplastic disease that must be differentiated. It is similar to erythroblastosis in that it is invariably leukemic but, as with visceral lymphomatosis, the neoplastic cell—the granuloblast—is of extravascular origin and it can arise also extramedullarly (figs. 13 and 14). This cell is on the average smaller than either the proerythroblast or the lymphoid stem cell, its cytoplasm is more acidophillic, and its shape is polygonal and angular; the nucleus is less vesicular, and the nucleolus, while present, is not nearly as frequently seen or as conspicuous as in the other two leukoses. The leukemia is spectacular in this disease. Grossly, terminal granuloblastosis is characterized by a moderately enlarged liver which is congested and studded with minute, circumscribed, grayish areas and a somewhat swollen, pale spleen.

It is quite evident that artificially induced visceral lymphomatosis appears to be in all respects similar to Ellerman's "lymphatic leucosis" (*38*) and to Campbell's "lymphoid leucosis" (*19*); however, the latter implied reservations concerning the transmissibility of these neoplasms are unfounded (*39*) and (*3*). Under Campbell's proposed terminology, "visceral lymphomatosis" is essentially a lymphogranulomatous inflammatory change in the viscera of birds with fowl paralysis (neural lymphomatosis). This terminology has been supported also by Darcel (*20*), whose view, however, that the bone marrow is unaffected in lymphoid leukosis appears to be at variance with Campbell who writes (*19*): "The leucoses are neoplastic conditions affecting haematopoietic tissue." Our own experience indicates that in both the artificially induced and the naturally occurring disease the bone marrow is one of the most regularly affected tissues; often the involvement is so extensive that it is grossly visible.

Osteopetrosis

In the field this disease is widespread, yet the incidence in individual flocks is usually very low. A comprehensive study of lesions in five naturally occurring cases, as well as positive results of transmission experiments, has been published by Jungherr and Landauer (*40*). In experimental birds it can coexist with erythroblastosis, visceral lymphomatosis, hemangiomatosis, or with hemangiomatosis and either of the first two. It was observed to arise following inoculation with virus from a variety of sources; yet certain strains have never elicited osteopetrosis, a fact which might be construed as indirect evidence for a distinct etiologic agent (*3*). The detailed incidence of osteopetrosis in serial passages with RPL12 cell-free material from individual donor birds is presented in the third paper of this series (*41*).

Pathogenesis experiments indicate that in the early phase of uncomplicated osteopetrosis the diseased birds have an anemic, unthrifty appearance. Many suffer from leg fractures and all the long bones can be easily crushed at necropsy. Microscopically, the first visible alteration is an increase in size and number of the cells in the deep layer of the periosteum, the endosteum, and in the haversian canals (fig. 10). This proliferation is rapidly progressing and assumes neoplastic features, as evident by the partial lysis of the compact bone (fig. 11).

Unless the diseased bird succumbs at this stage due to secondary infections and undernourishment as a result of lameness, this lytic process subsides and a new phase of endochondral osteogenesis (to a small degree concomitant with the lysis) becomes very evident. New cartilaginous tissue is laid down in the lytic spaces and mineralization follows (fig. 12), resulting in the formation of bone with an imperfect lamellar system. This regeneration proceeds well beyond the stage of merely replacing the osseous tissue that was lysed and its enduring effect is the most striking feature of this disease, even though it is probably secondary from the standpoint of pathogenesis. These lesions are limited to the diaphysis of the bone which becomes thick and hard ("marble bone"); the marrow cavity is partially or completely obliterated. Birds with this hypertrophic type of the disease do not appear to be as greatly affected, insofar as lameness and nutritional status are concerned, as are birds in the early phase. An interesting and frequent lesion that accompanies this syndrome is a markedly atrophic and fibrous spleen and, occasionally, a cirrhotic liver; the relation of these soft tissue changes to those undergone by the bone itself is not yet understood. Although osteopetrosis can be regarded as a neoplastic disease due to the extensive bone destruction by the proliferative process, the fact that this phase subsides poses some questions as to its malignancy. The osteogenic phase certainly cannot be classified as neoplastic in the sense of the bone-forming tumors of mammals or the osteochondrosarcoma described for the chicken (*42*). Considerably more study of osteopetrosis is necessary for a better understanding of the pathogenesis of this disease.

Hemangiomatosis

This manifestation is associated with certain passage series of RPL12 and RPL19 (*3*). It can be present by itself or jointly with erythroblastosis and/or osteopetrosis, and it is characterized by small, sharply circumscribed, dark-red, raised tumor nodules on the serosal surfaces of abdominal viscera (fig. 3), air sacs, and peritoneum. These nodules are mixed cavernous and capillary hemangiomas with the predominant cell of good differentiation (fig. 18). Frequently there are widespread linear hemorrhages in the voluntary musculature. The name "hemangiomatosis" previously suggested (*7*) correctly implies a multicentric rather than a local neoplasia.

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PLATE 15

FIGURE 1.—Erythroblastosis—liver; RPL12 inoculated. Acute passive hyperemia and gelatinous transudate covering right lobe.

FIGURE 2.—Visceral lymphomatosis—liver; RPL12 inoculated. Coalescing focal involvement.

FIGURE 3.—Hemangiomatosis—gizzard serosa; RPL12 inoculated. Dark, circumscribed, and raised tumor nodules.

Gross *et al.*

488412—59——13

PLATE 16

FIGURE 4.—Erythroblastosis—spleen; RPL12 inoculated. Intermediate stage: Distended sinusoids near artery are filled with neoplastic proerythroblasts; *note* undisturbed lymphoid focus, *center*, with a few immature cells.

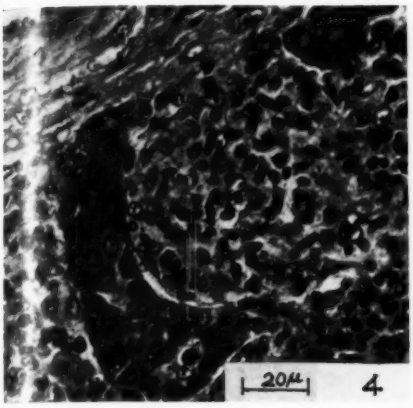
FIGURE 5.—Visceral lymphomatosis—bone marrow; RPL12 inoculated. Focal, circumscribed distribution of lymphoid neoplasm contrasts with the diffuse involvement of the marrow in erythroblastosis.

FIGURE 6.—Visceral lymphomatosis—bone marrow; RPL12 inoculated. Erythroid hyperplasia in central sinusoid between two lymphoid neoplastic areas.

FIGURE 7.—Erythroblastosis—liver; RPL12 inoculated. Terminal stage: marked distention of sinusoids with leukemic cells; *note* their uniformity in appearance and prominent nucleoli. *Compare* with figure 8.

FIGURE 8.—Visceral lymphomatosis—liver; RPL12 inoculated. Destruction of hepatic parenchyma by invasion of extravascular lymphoid neoplasm. These cells also have prominent nucleoli but are more variable in size and shape than the proerythroblasts (fig. 7).

FIGURE 9.—Visceral lymphomatosis—liver imprint smear; RPL12 inoculated. *Note* the large N/C ratio, the large nucleolus somewhat obscured, and the fine texture of the nucleus. The cells are variable in size and shape.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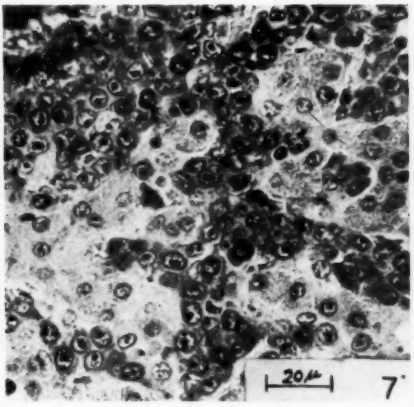
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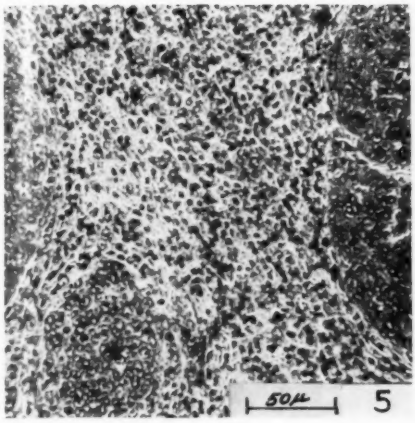
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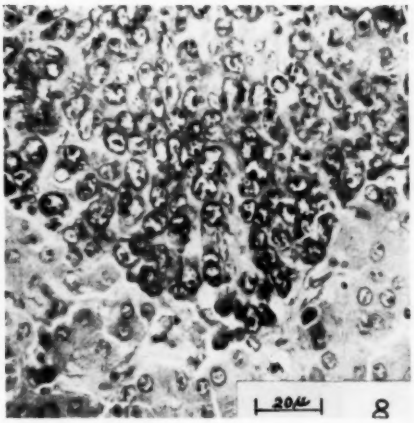
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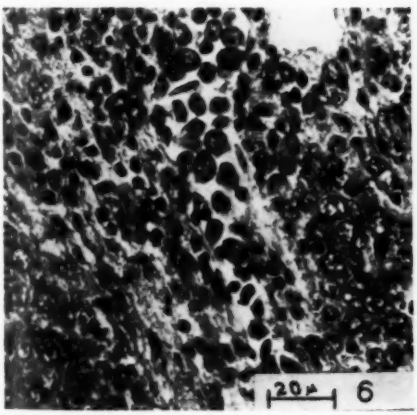
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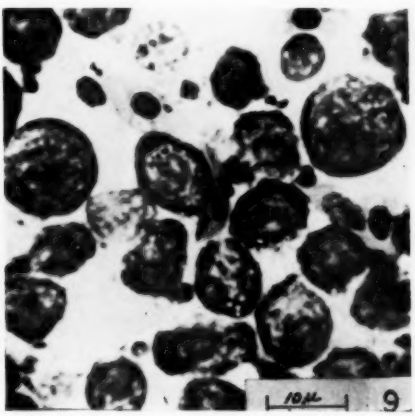
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PLATE 17

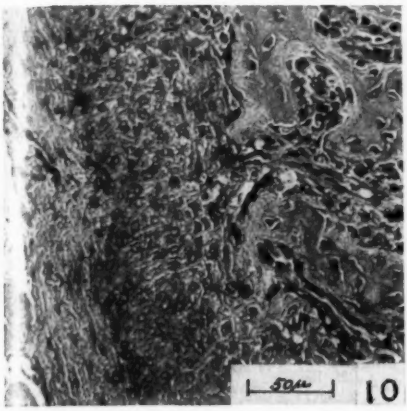
FIGURE 10.—Osteopetrosis—femur; RPL12 inoculated. Initial stage: increase in size and number of cells in deep periosteal layer and in the haversian canals.

FIGURE 11.—Osteopetrosis—femur; RPL12 inoculated. Osteolytic stage: The compact bone has a "Swiss cheese" appearance.

FIGURE 12.—Osteopetrosis—femur; RPL12 inoculated. Early osteogenic stage: A new cartilaginous spicule is being mineralized (denser areas). Osteoblasts and osteoclasts are visible.

FIGURE 13.—Granuloblastosis—liver; Beard's myeloblastosis virus inoculated; perivascular origin and distention of sinusoids by leukemic cells.

FIGURE 14.—*See* figure 13. *Compare* with figures 7 and 8 at same magnification and *note* smaller size of granuloblast, its irregular polygonal shape, lack of a conspicuous nucleolus, and the character of the chromatin clumping.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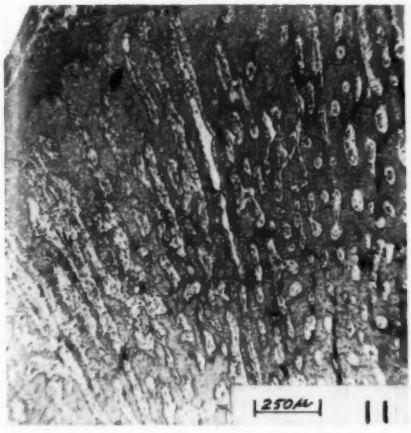
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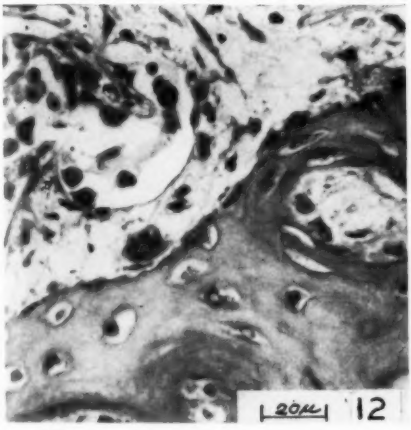
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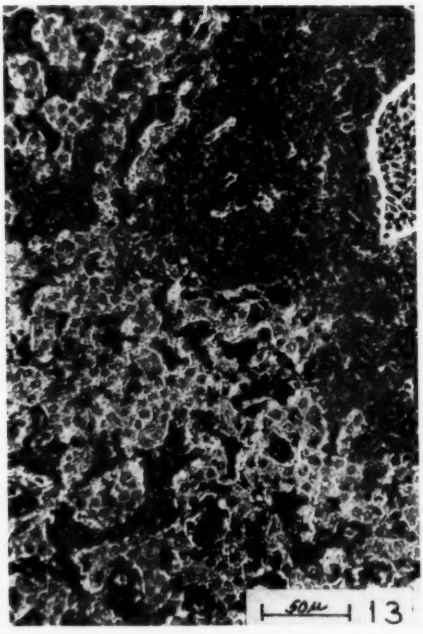
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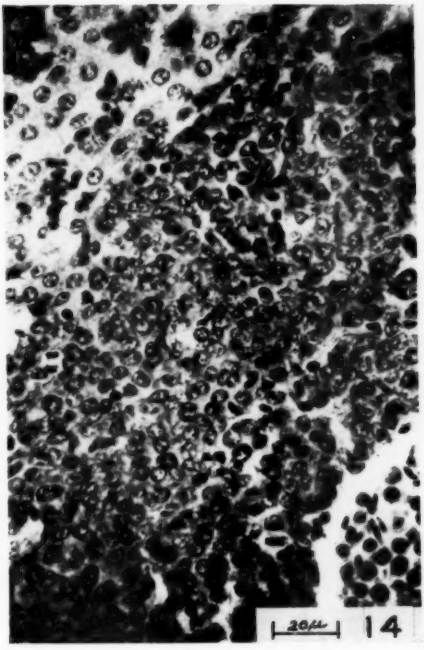
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PLATE 18

FIGURE 15.—Visceral lymphomatosis—liver. Naturally occurring case (original donor for RPL23). This bird had neoplastic cells in the circulating blood. *Note* high degree of anaplasia; remnants of hepatic parenchyma visible, *center*.

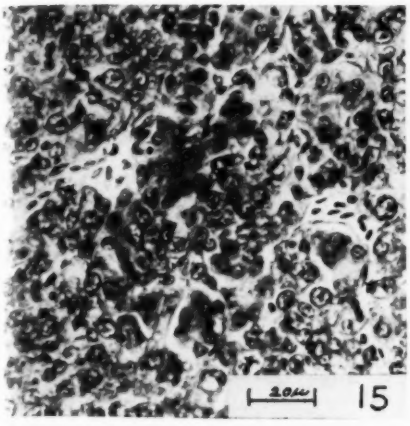
FIGURE 16.—Visceral lymphomatosis—liver. Naturally occurring case—intermediate malignancy; neoplastic cells at various stages of differentiation.

FIGURE 17.—Visceral lymphomatosis—liver. Naturally occurring case—benign. Cells are indistinguishable from mature lymphocytes.

FIGURE 18.—Hemangiomatosis—gizzard serosa; RPL12 inoculated (erythroblastosis coexistent). Same case as in figure 3. *Note* the caverns and capillaries as well as the marginated proerythroblasts.

FIGURE 19.—Neural lymphomatosis—abdominal vagus. Naturally occurring; chronic diffuse inflammatory reaction with plasma cells, monocytic macrophages, and lymphoid cells at different levels of differentiation.

FIGURE 20.—Focal thrombotic granuloma—liver. Naturally occurring (otherwise normal chicken). *Note* similarity between immature lymphoid cells in central necrotic area and those in malignant visceral lymphomatosis.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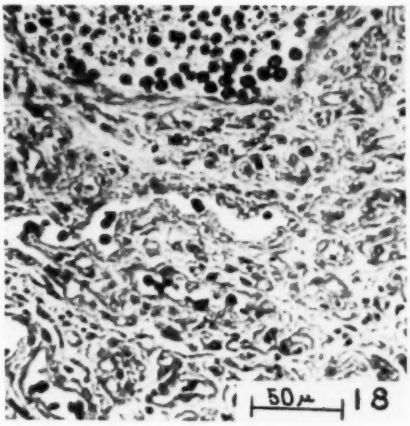
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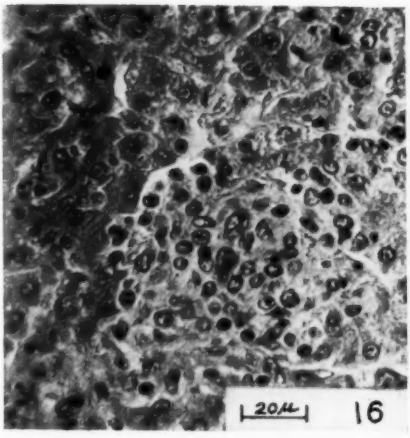
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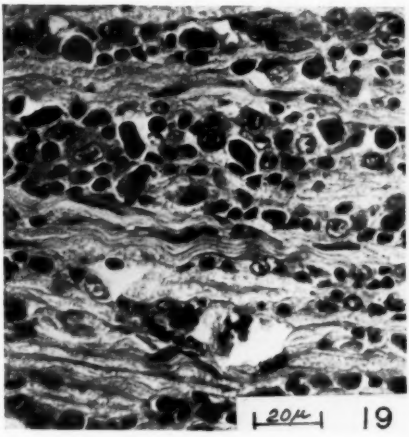
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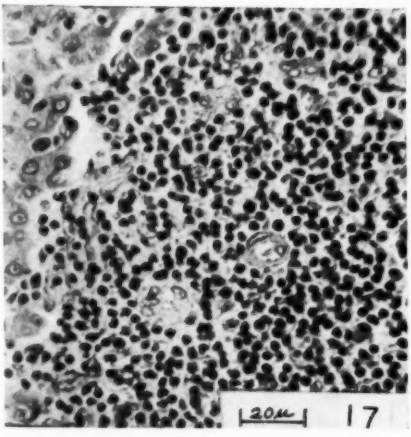
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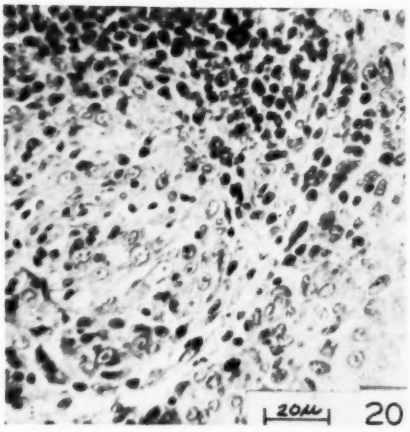
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Pathogenicity of a Viral Strain (RPL12) Causing Avian Visceral Lymphomatosis and Related Neoplasms. II. Host-Virus Interrelations Affecting Response [1]

B. R. Burmester, M. Adrian Gross, W. G. Walter, *and* A. K. Fontes, *United States Department of Agriculture, Agricultural Research Service, Regional Poultry Research Laboratory, East Lansing, Michigan*

Summary

A study was made of the disease-response in chickens experimentally exposed by different methods to various sources of virus or viruses causing visceral lymphomatosis and related neoplasms. All sources of virus that caused visceral lymphomatosis also caused erythroblastosis. These included tumors from 7 different naturally infected chickens from various sources, embryos from 28 different hens, as well as oral washings and fecal collections from 9 different hens. Filtrates of tumors and other materials from 5 of the 7 propagated lymphoid-tumor strains caused osteopetrosis in addition to erythroblastosis and visceral lymphomatosis. Hemangiomas and fibrosarcomas or myxosarcomas were also observed in chickens of certain strains and passages, but there were few myelocytomas or incidences of neurolymphomatosis, ocular lymphomatosis, and granuloblastosis. A variation in the dose of virus had a marked effect on the type of response. High doses primarily caused erythroblastosis, which occurred in less than 4 months, and low doses resulted in a response that was primarily visceral lymphomatosis, with death occurring after 4 months. Variations in the type and quantity of response following exposure by different methods (including contact) and to different sources of virus were obtained. These variations can be most logically explained on the basis of a variable level of effective virus.—**J. Nat. Cancer Inst. 22: 103–127, 1959.**

Filtrates of a lymphoid tumor, isolated and described by Olson (*1*, *2*) and extensively investigated at this laboratory as strain RPL12, caused visceral lymphomatosis and several related neoplasms (*3*, *4*). Transmission was also obtained with filtrates prepared from lymphomatous tumors from chickens of the breeding flock at the laboratory (*5*). In addition, visceral lymphomatosis was induced at high rates with cell-free inoculums prepared from normal-appearing liver of embryonic eggs, incubator debris, oral-cavity and respiratory-tract washings, and fecal extracts obtained from chickens of the genetic flock (*6–10*).

The disease was also induced in chickens reared in direct contact with pen mates that had been inoculated with the filtrable agent of strain

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488412—59——14

RPL12 (*11*). Several pathologic entities that occurred in the inoculated chickens have long been recognized (*12*). When "natural" sources of virus were used, such as filtrates of tumors from naturally occurring visceral lymphomatosis, embryonic liver, fecal extracts, and oral and respiratory-tract washings, the primary response was visceral lymphomatosis with death occurring after more than 4 months. This was characterized by the extravascular neoplastic proliferation of lymphoid cells in one or more viscera. Of secondary significance was the occurrence of an intravascular involvement with features resembling erythroblastic leukosis (*12*). The latter, however, became more prevalent after the causative agent(s) had been propagated for several generations in susceptible chickens. With large doses of filtrate prepared from donors that died early, a high proportion of the inoculated chickens died within 4 months and almost all of these had the intravascular form of the disease.

The distribution of the intravascular and extravascular forms was found to be related to the inoculation dose (*13*). Large doses resulted in a high proportion of the intravascular syndrome, which caused death early, whereas low doses resulted in a high proportion of extravascular lymphomatosis, with death generally occurring after 4 months. It was suggested (*12*) that the former might be the acute and the latter the chronic expression of the same disease, though the possibility that they were caused by different viruses was also pointed out. Since there were several similar histopathologic features and since the intravascular syndrome occurred in all inoculation experiments resulting in visceral lymphomatosis, it seemed appropriate to use only the distribution of neoplasia as a criterion for differentiation. Hence, the terms "intravascular" and "extravascular" visceral lymphomatosis were used (*13*). After extensive comparative studies, the intravascular disease now is recognized as erythroblastosis (*14*, *15*).

Data will be presented on the mortality from erythroblastosis and visceral lymphomatosis as it is influenced by such variable factors of the host as: age at inoculation, genetic constitution, or variation in conditions of exposure, *i.e.*, method, route, or, if by inoculation, source and dose of virus. Since the data were obtained from many different experiments, the description of the different procedures and protocols will be held to a minimum. In most instances the procedure and primary transmission results have been reported elsewhere.

Experiments and Results

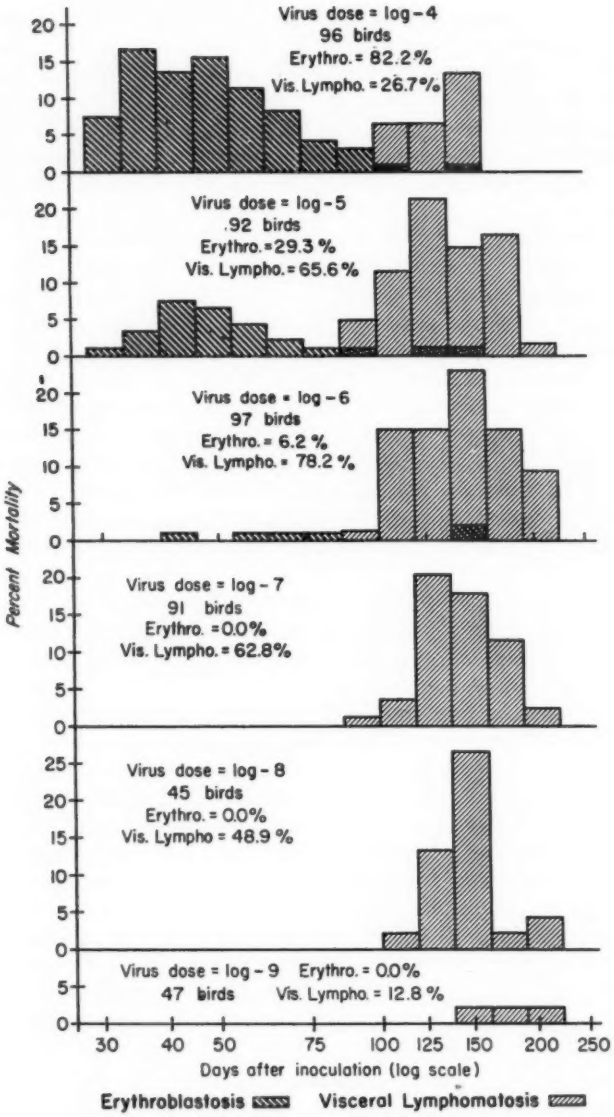
Influence of Dosage

The response of 4 groups of susceptible chickens to graded doses of the virus of strain RPL12 has been reported (*13*). The occurrence of the intravascular disease erythroblastosis was found to be directly related to dose, whereas the occurrence of visceral lymphomatosis in the same lots appeared to be inversely related to dose over the range tested. In 2 of the 4 titrations there were indications of a reversal in this latter trend for the lowest

dose tested. A more complete indication of response from a recent titration is presented in text-figure 1.

Six lots of chickens from a susceptible inbred line (*16*) were inoculated intravenously at 2 weeks of age with serial tenfold dilutions of preparation L31 of strain RPL12. This preparation was a filtered extract of livers from chickens of the sixteenth serial cell-free passage of tumor strain RPL12. The dosage is in terms of the log grams of tumor used in preparing the filtrated inoculum (*4*). Chickens of each dosage group were kept in separate cubicles until about 10 weeks of age (*17*), when they were moved to regular multilot pens and kept until 9 months of age.

The histogram of text-figure 1 depicts the proportion of birds that died of erythroblastosis and visceral lymphomatosis, respectively, during equal intervals of log days after inoculation. The period of 20 to 260 days after inoculation was divided into 16 equal intervals of 0.070 log days. The erythroblastosis or visceral-lymphomatosis mortality for each period was calculated from the number of birds inoculated less those that died of other causes before or during the experimental period of each disease. Thus, in calculating the percentage of erythroblastosis, all other deaths that occurred before or during the time interval within which deaths from erythroblastosis had occurred were subtracted from the total number inoculated. In calculating the visceral-lymphomatosis mortality, the erythroblastosis mortality was considered to be a different response; hence, the percentage of visceral lymphomatosis is based on the number of birds that were alive at the time visceral-lymphomatosis mortality started. This number is determined by subtracting from the total number inoculated all deaths due to secondary causes and also those due to erythroblastosis. There are several considerations that enter into this method of correcting for other deaths. It is obvious that when the correction is large, due to a high erythroblastosis mortality as in the log −4 dosage groups (text-fig. 1), the percentage figure for visceral lymphomatosis is increased greatly. Often this results in an apparently misleading combined erythroblastosis and visceral-lymphomatosis mortality of greater than 100 percent for any given lot. The primary reason for the use of this rather bold correction is that the mortality pattern and the morphologic alterations indicate that erythroblastosis and visceral lymphomatosis are distinctly different disease entities. Furthermore, since almost all erythroblastosis mortality occurs before the visceral-lymphomatosis mortality, chickens that die of erythroblastosis have no opportunity to die of visceral lymphomastosis; therefore, such chickens ought to be removed from the population when the visceral-lymphomatosis mortality is considered. Despite this correction, there is still a bias because the more susceptible birds are already dead and thus the population remaining after the erythroblastosis deaths is no longer a random one. If there is any relation between the innate resistance to erythroblastosis and to visceral lymphomatosis, the chickens surviving the onslaught of erythroblastosis are likely to be relatively more resistant than the original population to visceral lymphomatosis.



TEXT-FIGURE 1.—Influence of virus dose on mortality from erythroblastosis and visceral lymphomatosis.

The time-mortality histograms of text-figure 1 show that most of the chickens that had received a large (log —4) dose of virus died of erythroblastosis. The proportion of erythroblastosis deaths decreased markedly with each tenfold dilution of the inoculum, so that at log —6 only 6.2 percent

died of erythroblastosis. Conversely, the proportion of birds that died of visceral lymphomatosis increased to a maximum at a dose of log −6. The increase was most marked between the highest (log −4) and the next highest (log −5) dose. The low visceral-lymphomatosis mortality obtained in the highest dosage lot was undoubtedly due largely to the high resistance of the remaining 15 of the original 96 chickens at the time visceral lymphomatosis became manifest. The percentage of visceral lymphomatosis decreased rapidly within the dilute range of log −7 to log −9. These results are like those reported earlier (*13*) on the relative occurrence of the two diseases; however, here a positive dose-response correlation for visceral-lymphomatosis mortality was obtained in the region of very low virus dose.

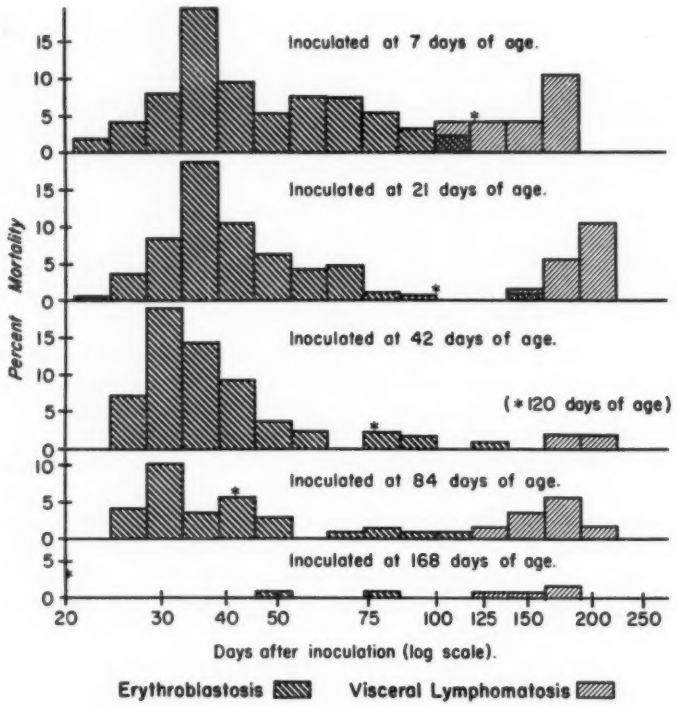
The data show that there was a marked difference in the host age at death due to each of the two diseases. Almost all the erythroblastosis mortality took place within the first 100 days after inoculation and most of the visceral-lymphomatosis mortality occurred subsequently. There were also two rather distinct peaks of mortality: the erythroblastosis peak at 40 to 50 days and the visceral-lymphomatosis peak about 100 days later. These time relations were quite consistent from group to group, irrespective of the dose in the large range from log −4 to log −8.

A similar separation of the time of incidence of the two diseases has also been obtained with other preparations of strain RPL12 in titrations at a dosage range of log −3 to log −8 (*13*). It was found that of 251 fatal cases of erythroblastosis, 92.8 percent died prior to 121 days after inoculation, and of 221 cases of visceral lymphomatosis, 93.7 percent died after 120 days.

Influence of Age at Inoculation

Two experiments on the influence of age at inoculation on the mortality of chickens will be reported here. In the first, a filtered extract of a tumorous liver of the eighteenth serial passage of RPL12 was inoculated by the intravenous route. In the second, a similar material of the sixteenth serial passage was given intraperitoneally. Methods of chicken maintenance, diagnosis, and analysis of mortality data were similar to those already described. Since this report is not primarily concerned with the dose-response relationship as affected by age, but with the influence of age on the time-to-death, measured in days after inoculation, and the relative time distribution of erythroblastosis to visceral-lymphomatosis deaths, the various dosage groups were combined. The total number inoculated and the weighted average dose for the 7-day and 21-day age groups of the first experiment were 200 chickens in each and a dose of log −3.5. For the 42-, 84-, and 168-day age groups there were 150 chickens and a dose of log −3.0. In the second experiment chickens in all age groups were inoculated with 3 tenfold dilutions of virus, which gave an average of log −4 and a total of 150 chickens for each age group.

The time-mortality data for the two diseases are presented in text-figures 2 and 3. These data also show two modes, an early one from erythroblastosis and a late one from visceral lymphomatosis. The two



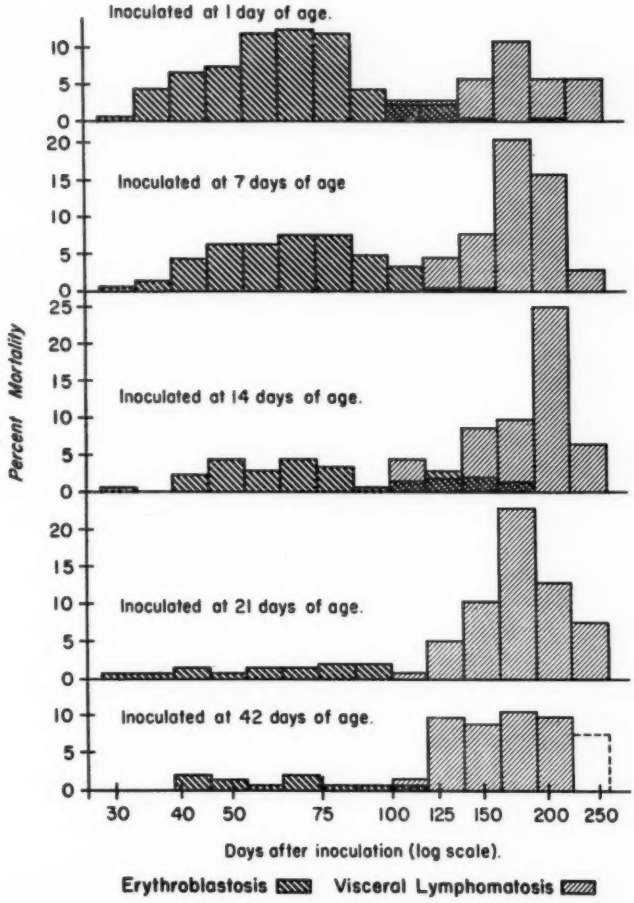
TEXT-FIGURE 2.—Influence of age at inoculation on mortality from erythroblastosis and visceral lymphomatosis.

peaks were separated by approximately 100 days, with only a slight overlap in the period of occurrence of the two diseases. Since inoculations in experiment 1 were made at age intervals up to 168 days, it was of interest to learn whether the transition from erythroblastosis to visceral lymphomatosis took place at a constant age of the chickens or at a constant period after inoculation.

The data of both experiments suggest that most of the birds with erythroblastosis died before they were 120 days of age, irrespective of the age at inoculation up to 84 days. There was a slight indication in the data of experiment 1 that the average time-to-death, measured in days after inoculation, decreased as the age of birds at inoculation increased. These relations indicate a distinct age susceptibility to erythroblastosis.

Although most birds with erythroblastosis died in less than 120 days of age, as the age at inoculation was advanced more birds died after 120 days. Similar indications were obtained with other sources of virus in which the virus concentration was expected to be low. These data are presented in a following section.

It would appear that the time-to-death for any given bird was a function of its age at the time of exposure and the dose of the virus. A con-


Inoculated at 1 day of age.
Inoculated at 7 days of age
Inoculated at 14 days of age.
Inoculated at 21 days of age.
Inoculated at 42 days of age.
Percent Mortality
Days after inoculation (log scale).
Erythroblastosis
Visceral Lymphomatosis

TEXT-FIGURE 3.—Influence of age at inoculation on mortality from erythroblastosis and visceral lymphomatosis.

sistent and significant result of both experiments was that the visceral-lymphomatosis deaths did not begin until after approximately 100 days post inoculation, irrespective of the age of the bird when inoculated. This suggests that the minimum incubation period for visceral lymphomatosis induced by strain RPL12 virus is about 100 days, though a significant incidence does not occur until after approximately 120 days.

Influence of Route of Inoculation

Data on the influence of the route of inoculation on the occurrence of erythroblastosis and visceral lymphomatosis were obtained from 3 experiments. The transmission data and the experimental procedures of

the first 2 have already been reported (*17*). In the third experiment, day-old chicks of isolated line 15 were given strain RPL12 virus (*13*) at 3 serial tenfold dilutions by the following routes: intravenous, intramedullar, intraperitoneal, intracranial, intramuscular, tracheal, subcutaneous, and nasal. Methods of chicken maintenance, diagnosis, and analysis of mortality data were similar to those employed for experiments reported in previous sections, and the 3 dosage lots for each route were combined for the same reasons and by the methods given.

Table 1 gives the essential data on the occurrence of erythroblastosis and visceral lymphomatosis, when the RPL12 virus was given by the several routes. All the various routes used, including the aerogenic, resulted in mortality from both erythroblastosis and visceral lymphomatosis; however, the effectiveness of the routes varied considerably, and there was also a variation in the relative proportion that died of erythroblastosis and visceral lymphomatosis. Such routes as intravenous, intramedullar, and intraperitoneal resulted in a high incidence of erythroblastosis. The percentage of visceral lymphomatosis in this route group was only moderate, since an intermediate-to-high proportion had already died of erythroblastosis. The moderately effective routes—intracranial, intramuscular, subcutaneous, and tracheal—resulted in intermediate levels of erythroblastosis, with a somewhat higher percentage of visceral lymphomatosis. Aerogenic and nasal routes resulted in a very low incidence of erythroblastosis and a moderate-to-high percentage of visceral lymphomatosis.

It would appear that none of the various routes tested was notably effective in producing only one or the other of the two diseases. Although within experiments a nearly constant dosage for the various routes was

TABLE 1.—*Influence of route of inoculation on mortality from erythroblastosis and visceral lymphomatosis*

Route of inoculation	Number inoculated	Response			
		Erythroblastosis		Visceral lymphomatosis	
		Number	Percent*	Number	Percent*
Experiment 1 and 2 (average log dose = −2.0)					
Intraperitoneal	77	58	84. 1	5	50. 0
Tracheal	80	17	23. 3	41	83. 7
Aerogenic	76	5	6. 6	43	64. 2
Experiment 3 (average log dose = −3.6)					
Intramedullar	139	60	46. 2	30	45. 5
Intravenous	146	51	37. 8	36	50. 0
Intraperitoneal	146	44	32. 6	47	52. 2
Intracranial	142	36	26. 5	56	58. 3
Intramuscular	146	35	25. 6	41	47. 7
Subcutaneous	143	23	17. 3	63	63. 6
Nasal	147	2	1. 4	20	14. 7

*Based on corrected number (*see* text).

employed, it is probable that certain routes, such as aerogenic and nasal, resulted in much loss of virus. Other routes, such as subcutaneous, probably resulted in loss by inactivation of virus before it could be transported to susceptible cells. Thus, in view of the marked influence of dosage on the relative occurrence of these two diseases, it is probable that the variations obtained with the different routes were primarily, if not wholly, due to variations in effective dose, *i.e.*, the amount of virus that has come in contact with the susceptible cells.

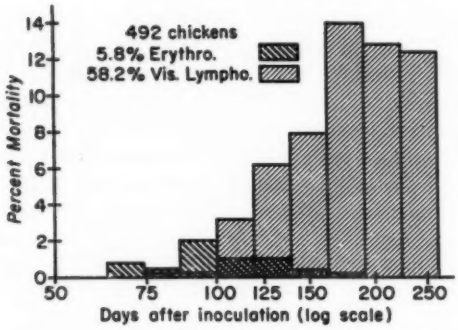
Influence of Sources of Virus

Washings obtained from the oral cavity alone, or from oral cavity and upper-respiratory passages, have been found to contain infective levels of virus. Infectious saline extracts of various fecal collections also have been found. Such virus levels have been demonstrated not only in material collected from chickens with large tumors, which resulted from natural sources of virus or following inoculation with RPL12 virus, but also in material from chickens that appeared normal but had virus from similar sources. In other experiments it was found that 15-day embryos of eggs laid by certain hens of a naturally infected flock contained infectious levels of virus. The individual quantitative results of these infectivity tests, together with a description of the materials and methods, have already been reported (*7–10*).

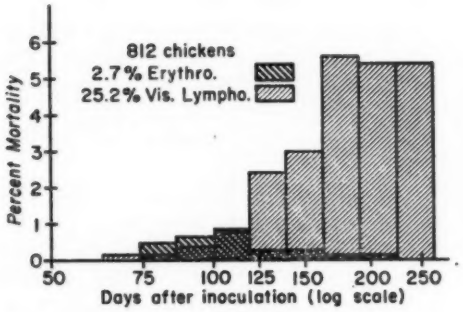
It was of interest to re-examine these data for the relative occurrence of erythroblastosis and visceral lymphomatosis. For this purpose, the previously mentioned infectivity tests were classified into 3 categories. There were 4 experiments involving a total of 492 birds that received oral washings or fecal extracts of chickens previously inoculated with strain RPL12 virus. The time-mortality data of this group are presented in text-figure 4. Text-figure 5 presents similar data from 4 other experiments involving a total of 812 chickens that received oral washings or fecal extracts of chickens naturally infected. A total of 2,042 chickens were inoculated with 15-day embryo-liver extracts obtained from eggs laid by naturally infected hens. The time-mortality data of these inoculations are presented in text-figure 6.

The histograms for the 3 infectivity tests are quite similar. Erythroblastosis and visceral lymphomatosis occurred in all inoculations. The proportion of erythroblastosis to visceral lymphomatosis was about 1:10 for each of the 3 groups, even though the percentage of visceral lymphomatosis varied from an average high of 58.2, for chickens inoculated with materials collected from strain RPL12-inoculated chickens, to a low of 25 percent for the 2 groups that received material from naturally infected hens. Deaths from visceral lymphomatosis were almost entirely confined to the period after 100 days, while erythroblastosis deaths extended over 60 to 200 days. The latter period is much longer than that obtained with many experiments where tumorous liver filtrates were used.

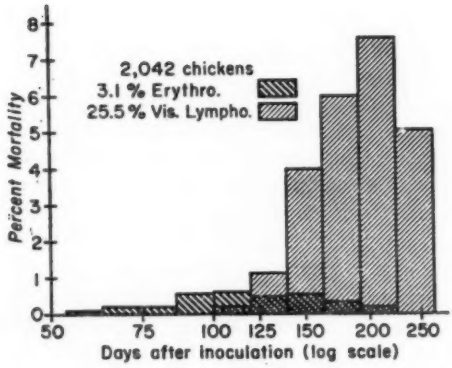
These data show that the virus which is shed under natural conditions



TEXT-FIGURE 4.—Time-mortality data after inoculation with oral washings or fecal extracts of strain RPL12-inoculated birds.



TEXT-FIGURE 5.—Time-mortality data after inoculation with oral washings or fecal extracts of naturally infected chickens.



TEXT-FIGURE 6.—Time-mortality data after inoculation with extract of 15-day embryo liver of normal hens.

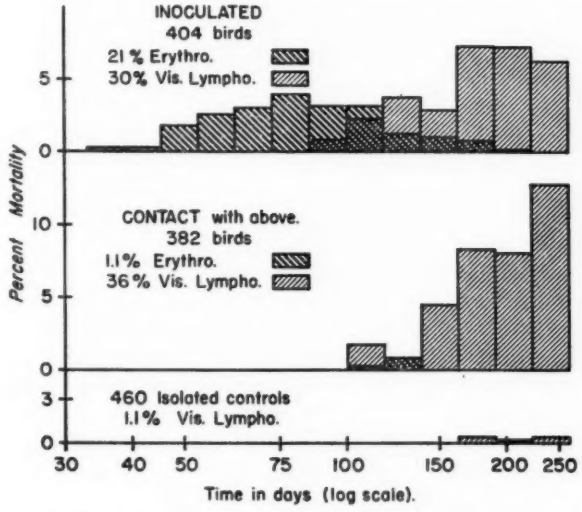
from the alimentary tract, or which is deposited in the egg and taken up by the embryo, is capable after intraperitoneal inoculation of causing not only much visceral lymphomatosis but also erythroblastosis.

Influence of Transmission by Experimental Contact

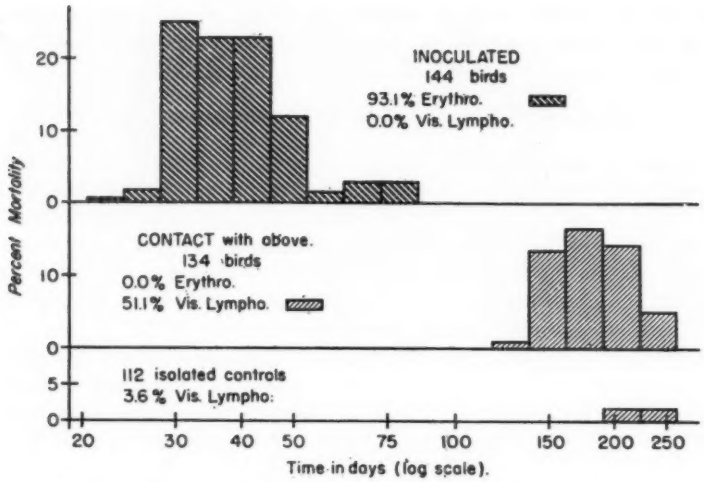
Visceral lymphomatosis can be transmitted from either naturally infected or inoculated chickens to noninfected pen mates of approximately the same age. The extent of such transmission by contact is conditioned by the exposure level, which in turn is influenced by the proportion of infected chicks and the length of exposure period (*11*). The time-mortality data of 3 such contact-transmission experiments with strain RPL12 were combined and are presented in text-figure 7. A total of 404 inoculated birds developed an average of 21 percent erythroblastosis and 30 percent visceral lymphomatosis. During the entire experimental period of 9 months, they were in direct contact with uninoculated chicks of the same inbred line. Of the 382 contact birds, 36 percent died of visceral lymphomatosis, but only 4 birds died of erythroblastosis.

The distribution of mortality with the time-to-death from erythroblastosis among the inoculated birds was similar to that in other RPL12-inoculated groups. The deaths due to visceral lymphomatosis, in both the inoculated and the contact groups, were almost entirely confined to the period after 100 days as in previous groups described. Only 5 of 460 isolated control birds succumbed to visceral lymphomatosis.

In a fourth contact-transmission experiment, only erythroblastosis was obtained in inoculated birds and only visceral lymphomatosis in contacts. These remarkable results are presented separately in text-figure 8. For



TEXT-FIGURE 7.—Combined time-mortality data of chickens in contact with strain RPL12.



TEXT-FIGURE 8.—Time-mortality data of chickens inoculated and others in contact with strain RPL12 virus.

this experiment chicks were inoculated intravenously at 1 day of age with a dose of log −4, and were reared together with an equal number of uninoculated chicks of the same hatch. The results show that 93 percent of the inoculated birds died of erythroblastosis in less than 83 days. Of the 134 contact birds, 51 percent died of visceral lymphomatosis during the period of 131 to 270 days of age.

This experiment clearly shows that although the source of infection, *i.e.*, the inoculated birds, showed evidence of only one disease, these same birds were the source of a different disease which developed in only the contact birds. There was a complete segregation of response between the inoculated and uninoculated contact chickens, not only with respect to the type of the disease causing death but also to the age or to the period after exposure when death occurred.

Influence of Genetic Constitution

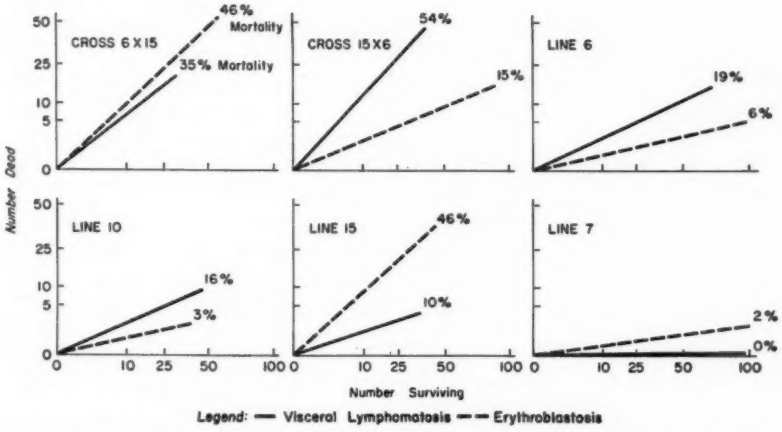
Several inbred lines of White Leghorn chickens selected for resistance or susceptibility to lymphomatosis have been developed, and mortality under natural conditions of exposure described (*18*, *19*). Responses obtained after inoculation with strain RPL12 have shown that, among lines and interline crosses and among dam families within lines or crosses, there was a significant variation in the percentage of chickens that developed lymphomatosis. A highly significant coefficient of correlation between the responses of families naturally exposed and their sibs after inoculation was also observed (*20*).

In view of the more recent interpretation of the response of chickens inoculated with strain RPL12, it was of interest to examine each line and

cross with respect to the proportion that died of erythroblastosis and visceral lymphomatosis.

Pedigreed chicks, hatched in 1951, were inoculated intraperitoneally, at 1 day of age, with undiluted preparation. The latter was obtained from livers of birds of the eleventh serial cell-free passage of tumor strain RPL12 by methods similar to those described (*13*).

The mortality to 300 days of age from visceral lymphomatosis or erythroblastosis that occurred in the inoculated chickens of 4 inbred lines and 2 interline crosses is shown in text-figure 9. The data were plotted on binomial probability paper,[2] with the number surviving the inoculation as the abscissa and the number that died as the ordinate.

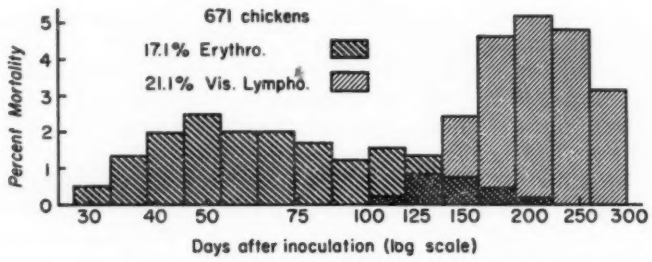


TEXT-FIGURE 9.—Response of chickens of several inbred lines and crosses to inoculation with strain RPL12 virus (binomial probability plot): — = percentage that died of visceral lymphomatosis; _ _ _ _ = percentage that died of erythroblastosis.

The graphs show that there was a great variation, not only among lines and crosses in the percentage of birds that died of erythroblastosis or visceral lymphomatosis but also in the ratio of erythroblastosis deaths to visceral-lymphomatosis deaths. Thus, the progeny of cross 6 × 15 had a high death rate from both erythroblastosis and visceral lymphomatosis. At the other extreme, line-7 chickens showed much resistance to both diseases. Of greater interest was the fact that chickens of the 15 × 6 cross had a high visceral-lymphomatosis and an intermediate erythroblastosis mortality, whereas line 15 showed a high erythroblastosis mortality with a low intermediate visceral-lymphomatosis mortality.

The apparent lack of pattern in the erythroblastosis and visceral-lymphomatosis mortality interrelationship suggests that the occurrence of visceral lymphomatosis is not closely related to the occurrence of erythroblastosis. This apparent lack of relationship indicates either that there are two distinctly different causative agents or that the genetic

[2] Paper No. 31,298 obtained from Codex Book Co., Inc., Norwood, Mass.



TEXT-FIGURE 10.—Combined time-mortality data of chickens of several inbred lines and crosses after inoculation with strain RPL12 virus.

resistance factors operate with little mutual dependence against the development of each of the two diseases rather than against establishment of infection. Text-figure 10 shows the distribution of deaths from erythroblastosis or visceral lymphomatosis among all the 671 chickens of the 4 lines and 2 crosses during the experimental period to 300 days of age.

Although most of the erythroblastosis deaths occurred before 120 days of age, some died as late as 184 days, whereas of those that developed visceral lymphomatosis all died after 100 days. This time-mortality response is much the same as that obtained with the isolated line-15 chickens after inoculation with natural sources of virus, such as oral washings, fecal extracts, and normal embryo-liver preparations.

Pathologic Manifestation of Filtrable Lymphoid-Tumor Strains During Early Serial Passage

The development and characteristics of several avian lymphoid tumors originating in field cases of visceral lymphomatosis have been described (*1, 2, 21, 22*). All these strains have been maintained in serial passage in chickens by the transfer of cellular suspensions at intervals of 1 to 3 weeks. Over half the strains were shown to contain a filtrable infectious agent which caused neoplasms, certain types of which were predominant (*3, 4, 5, 12*). In the early reports the intravascular disease was included with visceral lymphomatosis under the category of "visceral tumors." However, since this is considered a different disease from visceral lymphomatosis, it was of compelling interest to re-examine these transmission data for a pattern in the relative occurrence of the two diseases in the various strains studied.

The essential information relative to the methods, materials, and animals employed has already been reported for all strains except RPL23. This strain was recently isolated from field cases of visceral lymphomatosis in Ohio. Methods and materials used in the study of this strain and of other strains are similar in all respects to those reported earlier. Methods employed in the presentation of the responses are the same as those used on data given earlier in this paper.

Table 2 summarizes the essential data on the occurrence of visceral lymphomatosis, erythroblastosis, and osteopetrosis in strains RPL12,

TABLE 2.—*Summary of transmission data obtained in tests for virus in the early serial passage of several strains derived from naturally occurring cases of visceral lymphomatosis**

Source of inoculum	Serial cell-free passage	Number inoculated	Response					
			VL		E		Osteopetrosis	
			Number	Percent†	Number	Percent†	Number	Percent†
Strain RPL12								
59th transplant passage	1	28	21	78	0	0	12	43
65th " "	1	28	13	50	2	7	8	29
85th " "	1	29	18	64	0	0	12	41
VL donors	2	47	34	78	0	0	13	28
	3	32	22	76	0	0	16	50
	4	27	18	67	0	0	4	15
	5	14	4	29	0	0	2	7
	6	19	17	90	0	0	15	79
E and VL donors	7	23	9	45	4	17	2	9
	8	7	5	100	2	29	1	14
	9	30	17	68	8	27	10	33
	10	48	12	44	21	44	2	4
Strain RPL16								
3rd transplant passage	1	28	7	33	7	25	0	0
VL donors	2	29	13	65	9	31	1	3
	3	24	14	61	1	4	1	4
	4	19	4	25	3	16	3	16
E donors	1	18	16	89	1	6	0	0
	2	29	4	40	19	66	0	0
	3	25	0	0	10	40	0	0

See footnotes at end of table.

TABLE 2.—*Summary of transmission data obtained in tests for virus in the early serial passage of several strains derived from naturally occurring cases of visceral lymphomatosis**—Continued

Source of inoculum	Serial cell-free passage	Number inoculated	Response					
			VL		E		Osteopetrosis	
			Number	Percent†	Number	Percent†	Number	Percent†
Strain RPL18								
Originating donor	1	15	11	92	0	0	9	60
VL donors	2	17	13	80	1	6	1	6
	3	20	19	95	0	0	0	0
3rd transplant passage	1	17	11	79	3	18	1	6
7th " "	1	10	7	78	3	30	0	0
17th " "	1	17	14	100	1	6	0	0
21st " "	1	18	12	100	6	33	0	0
Strain RPL19								
							Hemangioma	
Originating donor	1	15	5	63	5	39	0	0
Hemangioma-E donors	2	13	3	25	2	15	2	15
	3a	19	0	0	17	89	10	54
	4a	17	0	0	17	100	17	100
			(Same for passage 5, 6, 7)					
E only donors	3b	12	0	0	12	100	0	0
	4b	12	0	0	12	100	0	0
			(Same for passage 5, 6, 7)					
Strain RPL20								
							Osteopetrosis	
Originating donor	1	16	6	43	2	13	0	0
5th transplant passage	1	15	12	92	2	13	0	0
6th " "	1	29	19	65	0	0	0	0
VL donor	2	27	9	60	13	48	0	0

Strain RPL21								
Originating donor	1	20	0	0	0	0	0	0
6th transplant passage	1	27	21	85	1	37	22	82
Strain RPL23								
Originating donor #1	1	42	10	40	9	21	3	7
" " 2	1	21	3	16	2	10	0	0
" " 3	1	37	13	47	9	24	0	0
" " 4	1	23	2	22	5	22	1	4
VL donor	2	33	19	66	4	12	12	36
	3a	20	6	32	1	5	3	15
E donor	3b	23	4	50	15	65	1	4
	4b	36	3	13	25	70	2	3
	5b	65	6	30	40	62	11	6

*E = erythroblastosis; VL = visceral lymphomatosis.
†Based on corrected number (*see* text).

16, 18, 19, 20, 21, and 23 after intraperitoneal inoculation with cell-free material.

Data on strain RPL12 show that chickens inoculated with filtrates of the 59th, 65th, and 85th transplant passages and with the first 6 filtrate-passage materials were essentially free of erythroblastosis. Only 1 of 224 chickens developed erythroblastosis, whereas 29 to 90 percent developed visceral lymphomatosis. At the seventh passage, 4 birds had erythroblastosis, and 1 of these, together with a chicken with visceral lymphomatosis, was used to supply inoculum for the subsequent passages. A similar pooling mixture of donor material was used for the next 2 passages when the percentage of erythroblastosis increased. Subsequent passages were made with donors having only erythroblastosis and, in general, the percentage of occurrence of erythroblastosis continued to increase to a point where undiluted inoculum had caused all or almost all birds to die of erythroblastosis.

Osteopetrosis was observed in all groups inoculated with filtrates or other cell-free inoculations with this tumor strain. The data indicate that the transplantable lymphoid-tumor strain RPL12 carries a filtrable agent (or agents) which causes visceral lymphomatosis and osteopetrosis, and that only after several serial passages with cell-free materials did this strain acquire the ability to cause erythroblastosis.

The appearance of erythroblastosis at the seventh passage may have been due to: 1) increased concentration of a visceral-lymphomatosis virus, 2) increased concentration of a latent erythroblastosis virus, or 3) a mutation of a visceral-lymphomatosis virus to one causing erythroblastosis. These are probabilities to be considered, but are not subject to determination at this time.

Two short series of serial cell-free passages were made with strain RPL16 and both were started with the third transplant passage. In one series only donors with visceral lymphomatosis were used. The resulting incidence of visceral lymphomatosis ranged from 25 to 65 percent. Erythroblastosis also occurred in all 4 passages, and osteopetrosis in all but the first. In the other series only birds with erythroblastosis were used as donors. The visceral lymphomatosis decreased from 89 to 0 percent and erythroblastosis increased from 6 to 66 and 40 percent for the second and third passages, respectively.

Cell-free material of the originating donor of strain RPL18 caused 92 percent of the chickens to die of visceral lymphomatosis. This transmission level was approximated for 2 subsequent passages; out of 37 birds only 1 developed erythroblastosis. In addition, filtrability tests were made on tumors collected from the 3rd, 7th, 17th, and 21st tumor-cell transplant passages. A high incidence of visceral lymphomatosis was obtained, but a significant percentage of the birds also died of erythroblastosis.

Inoculation of 15 birds with the tumor extract from the case of naturally occurring visceral-lymphomatosis originating in strain RPL19 caused 5 birds to develop visceral lymphomatosis, and 5 to develop erythroblastosis.

One of the latter also had hemangiomas and this bird was used as source of inoculum for the next passage. Similar donors were used for the subsequent 5 passages with the result that by the fourth passage, and in the subsequent 3 passages, all birds died with a combination of erythroblastosis and hemangiomas. A second series of passages was begun with a second-passage bird showing only erythroblastosis, and was continued with donors having only this disease. All birds that died in this series showed evidence of erythroblastosis only.

Tests for the presence of virus were made on tumors originating in strain RPL20, on tumors of the fifth and sixth transplant passages, and on tumors of the first cell-free passage. All inoculations caused a high incidence of visceral lymphomatosis, and 3 of the 4 inoculations caused a certain amount of erythroblastosis.

Strain RPL21 was tested in the first passage and gave no evidence of a filtrable agent causing any neoplasms. Similar tests of tumors from the sixth transplant passage caused a high incidence of visceral lymphomatosis, erythroblastosis, and osteopetrosis.

Strain RPL23 was isolated from specimens obtained from an Ohio flock experiencing heavy losses from visceral lymphomatosis. Four typical lymphomatous birds were obtained, and inoculations made with filtered tumor extracts. In addition, passages 2, 3b, 4b, and 5b were made with erythroblastosis donors and passage 3a was made with a visceral-lymphomatosis donor. It is apparent that inoculums from all 4 of the original donors caused both visceral lymphomatosis and erythroblastosis. Both of these diseases, as well as osteopetrosis, occurred in all subsequent passages. The selection of birds with erythroblastosis as donors resulted in an increase in both the absolute and relative occurrence of this disease in recipients, with the occurrence of visceral lymphomatosis remaining at the same level.

Of 10 transplantable lymphoid-tumor strains propagated from field cases of visceral lymphomatosis, 7 were found to contain a filtrable agent or agents. However, the negative transmission results obtained with 3 strains—RPL14, 15, and 17—in the rather limited number of tests conducted are, in themselves, not conclusive as to whether virus was present. Tumors of other transplant passages may well have given positive results as was the case with strain RPL21 where the first passage resulted in no neoplasms and tumor extract of the sixth transplant passage caused a high incidence.

The 7 filtrable strains had several features in common. All 7 strains caused viseral lymphomatosis and erythroblastosis in one or more transmission experiments. With 5 of the 7, erythroblastosis occurred in the first test in which there was also some visceral lymphomatosis. Strain RPL18 showed the first occurrence of erythroblastosis in the second filtrate passage and in RPL12 its occurrence was delayed until the seventh passage. Strain RPL19 showed a definite tendency to lose its ability to cause visceral lymphomatosis as the incidence of erythroblastosis increased with serial passage. The much greater virulence of this strain,

causing most or all birds to die of erythroblastosis before they could die of visceral lymphomastosis, may determine this result. It would be of interest to know whether or not RPL19 would produce the same shift in responses after serial dilution of the virus as has been found with RPL12.

Osteopetrosis was also a common manifestation of these tumor strains. It occurred to a highly variable extent in one or more trials with 5 of the 7 strains. Hemangiomas occurred primarily in RPL19, and also appeared in one passage of RPL20, as well as in a series of RPL12 not presented in table 2.

Discussion

Data have been presented which clearly demonstrate a close relation between the dose inoculated and both the relative and absolute occurrence of the 2 related diseases—erythroblastosis and visceral lymphomatosis. Large doses of strain RPL12 virus regularly produced a high incidence of erythroblastosis with only little or no visceral lymphomatosis; for any given preparation, as the dose was decreased by serial dilution, the incidence of erythroblastosis decreased and that of visceral lymphomatosis first appeared to increase and then, with further dilution, decreased.

There was almost a complete separation of the two diseases with respect to time of occurrence. In the dose-response experiments, almost all erythroblastosis deaths occurred in less than 100 days, with a peak at about 50 days, and almost all visceral-lymphomatosis deaths occurred after this time, with a peak at about 150 days. In other experiments the separation of the two diseases with respect to time was not as obvious. Although almost all birds with erythroblastosis died by the time they were 4 months old, age does not entirely limit the occurrence of erythroblastosis. Thus, although the results of experiments involving a variation in the age at inoculation (text-figs. 2 and 3) also show that most birds with erythroblastosis died before they were 120 days of age, as the age at inoculation was increased a higher proportion of birds with erythroblastosis died after this age; this indicates that advanced age by itself may not be primary in limiting the development of erythroblastosis.

The length of the incubation period (or more precisely, the number of postinoculation days to death from erythroblastosis) appears to be subject to considerable variation. Many factors affect the rate of progression of the disease. The more important are susceptibility of the host, virus dose, and the virulence of the strain of virus. The response of several groups of isolated line-15 chickens to several preparations of strain RPL12 virus has been summarized. The time-to-death from erythroblastosis for the 10th, 50th, and 90th percentile was 35, 50, and 85 days, respectively. Similar measurements on groups of the same line of chickens inoculated with natural sources of virus, such as oral washings, fecal extracts, and normal embryo-liver extracts, was 70, 105, and 160 days, respectively. For the same inoculations, the time-to-death from visceral lymphomatosis was also estimated for the 10th and 50th percentile.

For the strain RPL12-inoculated group they were 120 and 165 days, and for the natural sources of virus, 135 and 190 days, respectively.

Virus of strain RPL12 was administered by the intravenous, intramedullar, intraperitoneal, intracranial, subcutaneous, tracheal, nasal, and aerogenic routes. Both erythroblastosis and visceral lymphomatosis resulted by all of these routes of exposure, and there was no indication that route *per se* was of outstanding influence on the occurrence of one or the other of the two diseases. The data indicate that the variations in the absolute and relative incidence of erythroblastosis and visceral lymphomatosis could be explained on the basis of the marked influence of dosage (text-fig. 1).

Uninoculated susceptible chickens reared in direct contact with others of the same age that were inoculated with strain RPL12 died at a high rate, primarily of visceral lymphomatosis. This has occurred regularly, even though most of the inoculated birds in one experiment died of erythroblastosis. Although most birds that acquire infection by contact develop visceral lymphomatosis, it cannot be said that erythroblastosis never results from contact infection since 4 of 382 birds in contact did develop this disease (text-fig. 7).

This low erythroblastosis–high visceral-lymphomatosis response observed in contact birds could very well be explained on the basis of known dose-response relationships (text-fig. 1). The mechanism involved in the transfer of virus in contact transmission is not fully known. However, the amount of virus effectively transmitted is probably quite small.

Infectivity tests of oral washings and fecal extracts collected from birds inoculated with strain RPL12 have given a similar result, *i.e.*, primarily visceral lymphomatosis (text-fig. 4). A similar response was obtained when large doses of virus were administered by the oral and nasal routes (table 1).

Dose appears to be the most important extrinsic operative factor governing the expression of erythroblastosis and visceral lymphomatosis. However, since in these experiments the explanation is based on inference, one cannot unequivocally conclude that the difference in response between inoculated and contact chickens is only because of differences in dose of virus.

Inoculations with strain RPL12 virus resulted in the occurrence of erythroblastosis in all of the 4 inbred lines and 2 crosses tested. Visceral lymphomatosis occurred in all these sources of chickens with the exception of line 7. Under the same conditions of exposure with respect to inoculum preparation, host age, dose, route of inoculation, and the experimental period, a wide range in the pattern of response was obtained (text-fig. 9). One cross had a high death rate from both erythroblastosis and visceral lymphomatosis, another had high visceral lymphomatosis and low erythroblastosis; line 15 had a high erythroblastosis and a low visceral lymphomatosis; and line 7 had low erythroblastosis and visceral lymphomatosis. There was no constant proportion in the occurrence of erythroblastosis and visceral lymphomatosis. The data thus indicate that

either the two diseases are caused by different viruses or, if by one virus, the individual genetic susceptibilities (or sensitivities) of the erythroid and lymphoid cell lines to infection and neoplasia vary with little mutual dependence. The expression of host susceptibility and/or resistance would thus be at the cellular level instead of at the individual host level, the latter implying a barrier against the establishment of an infection. A comprehensive and analytical discussion of this subject has recently been presented (*23*).

However, it should be borne in mind that data presented earlier on the influence of dose suggest a lack of independence between the genetic susceptibility to erythroblastosis and to visceral lymphomatosis. Were the two independent, one would expect, in the high dosage lots, more chickens, remaining after the erythroblastosis mortality is over, to die of visceral lymphomatosis than were actually observed. Since this did not occur, the hypothesis of independence between the two susceptibilities should be ruled out.

In addition to strain RPL12, many other sources of visceral lymphomatosis virus have been investigated. Embryonic eggs of normal-appearing hens from a naturally infected flock, oral washings, and fecal samples taken from hens with or without tumors have all caused both erythroblastosis and visceral lymphomatosis in susceptible chicks. The relative distribution of the erythroblastosis and visceral-lymphomatosis responses obtained with undiluted preparations were similar to those obtained with the most virulent preparations of strain RPL12 after these filtrates were diluted 1 : 10,000. Thus, the response obtained with these natural sources of virus was very closely simulated with materials of the sixteenth serial passage of RPL12 by making appropriate dilutions of the inoculum.

Cell-free inoculums prepared from tumors of naturally occurring cases of visceral lymphomatosis or from tumors of transplant passages of strains derived from naturally occurring cases, as well as sources of virus mentioned in the foregoing paragraph, have all caused a spectrum of pathologic alterations similar to that obtained in several passages of strain RPL12 (*14*).

It would seem highly significant that all sources of virus giving any response have caused both visceral lymphomatosis and erythroblastosis when factors such as dose and holding period were appropriate. Osteopetrosis was common in 5 of the 7 strains tested; however, it did not occur in chickens inoculated with oral washings, fecal extracts, or embryo extracts of naturally infected hens. A fourth disease entity, hemangiomas, occurred at high rates in certain passages of 2 of the strains (RPL12 and 19). Other tumors, such as fibrosarcomas and myxosarcomas, occurred sporadically in passages of various strains and their rate of occurrence was much higher than expected in uninoculated chickens.

Neural lymphomatosis and ocular lymphomatosis, generally included in the avian leukosis complex (*24*), are considered closely related to visceral lymphomatosis; however, the fact that they did not occur in these transmission experiments was conspicuous. Of several thousand chickens

involved in the experiments reported, none was found with ocular lymphomatosis and most inoculations were without the associated occurrence of neural lymphomatosis. In the few experiments in which neural lymphomatosis appeared, the incidence was low and could not be unequivocally attributed to the inoculum.

The data indicate that conditions necessary for the development of ocular and neural lymphomatosis were not present in these experiments. That the chickens of line 15 are susceptible to the neural disease is indicated by its occurrence in this line under other exposure conditions (*25*, *26*). No information on susceptibility to the ocular disease is available. Since the chickens may be considered susceptible to neurolymphomatosis and since this disease was conspicuously absent in experiments with virus from many sources giving rise to a high incidence of erythroblastosis and visceral lymphomatosis, it would appear likely that neural and visceral lymphomatosis are not caused by the same virus. Such interpretations have been made previously by a number of workers (*5*, *6*, *8*, *27*, *28*).

Of primary importance in this paper is the question of whether there is a specific etiologic agent in erythroblastosis and in visceral lymphomatosis or a common agent in both. There is no question that they should be classified as two distinctly different pathologic entities. In erythroblastosis the erythroid cell line is affected, the neoplastic cells are located within the vascular bed only, and the incubation period is less than 100 days; by contrast, in visceral lymphomatosis the lymphoid cell line is affected, the neoplastic cells are located primarily extravascularly, and the incubation period is almost invariably more than 100 days. These diseases have been defined and characterized by several investigators (*14*, *29*).

Although biological causes of disease are generally very specific, it is known that certain viruses under different conditions will provoke different pathologic entities (*30*). It has been suggested (*31–33*) that all forms of avian leukosis and also certain related neoplasms are caused by a single multipotent virus. However, evidence in support of this concept is not conclusive. A similar situation has been observed in the virus-induced leukemias and related neoplasms of the mouse. Several investigators have found that leukemia as well as several solid tumors could be induced in mice and other animals by the injection of cell-free filtrates (*34–39*). Whether a multipotent virus or several specific agents are involved in the causation of the spectrum of neoplasms obtained has not been determined; however, the term "polyoma virus" has become associated with these findings.

If the two diseases, erythroblastosis and visceral lymphomatosis, are each caused by a specific virus, there is little doubt that these agents are very closely related and similar in all the respects studied. It has been suggested (*40*) that the visceral-lymphomatosis virus may well be the "wild type" since, in contrast to erythroblastosis, this disease has maintained itself in most poultry populations for several decades. Viruses

causing related neoplasms could thus be variants or mutants of the visceral-lymphomatosis virus.

Analysis of the data presented does not appear to lead one to favor the acceptance of one hypothesis over the other. With respect to osteopetrosis and hemangiomatosis, however, the fact that these appear to be associated only with certain strains would favor the specific virus concept.

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Quantitative Biological Experimentation in the Virus and Cancer Fields [1]

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Summary

Some factors in the further development of quantitative biological methodology in virus and cancer research are discussed. Current concepts underlying the quantitative approach were reviewed, and an analysis was made of some of the interpretations of dose-response data in these medico-biological fields.—J. Nat. Cancer Inst. 22: 129–159, 1959.

Among the most fundamental advances of modern times in the biological sciences have been the development of statistical methods applicable to small samples and the construction thereupon of biometric methods that are the precision tools of present-day experimental biology. Although many investigators contributed to this new branch of science, the now-classical studies of W. S. Gossett (who used the pseudonym "Student") and Sir Ronald A. Fisher served as the foundation (*1–3*). Essential for investigating the reactions of living matter to various types of stimuli are the biometric procedures applicable to dose-response relationships, which, because of the problems they were initially applied to, are commonly referred to as "methods of biological assay." These methods have reached a high state of development in their application to biological standardization in the pharmaceutical industry, and as research tools in experimental pharmacology and physiology. Only within recent years, however, has their use and value for quantitative biological experimentation in the virus and cancer fields been fully realized.

Two general types of experimental problems which involve the reactions of living tissues to viruses and carcinogenic agents are 1) the use of specific reactions as biological indicators for measuring agent activity (*i.e.*, bioassay) and 2) investigations on the nature of the reactions themselves as part of a disease process or over-all host-agent interaction. The biometric tools for the study and analysis of dose-response relationships are applicable to both general problems.

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In the field of chemical carcinogenesis there is little need of bioassay, at present, because most of the active compounds can be purified and measured by chemical or physical methods. The application of dose-response analyses to chemical carcinogens is therefore largely for the purpose of studying the nature of the cancerous response itself. Although chemical carcinogens do not multiply as do viruses, in the lesions they induce, the response to carcinogens has much in common, biometrically, with the response to viruses because both agents are active in extremely minute quantities and both are dependent upon the proliferation of biological elements, *i.e.*, "amplification" (*4*), for the detection of reactions to the minute doses.

The measurement of virus activity, on the other hand, is still largely dependent upon biological assay, and both problems mentioned must be dealt with in experimental work on viruses.

This paper presents a discussion of some uses that have been made of biometric methods in cancer and virus research, and indicates some problems that biologists and biometricians must investigate to further develop quantitative biological methodology in these medico-biological fields. "Amplified responses" resulting from the multiplication of other types of biological agents, such as bacteria and microorganisms in general, present analytical problems like those considered here. Important contributions to common biometric methodology have already come from these allied fields (*5–7*).

Mechanisms of Amplification of Responses to Small Doses of Viruses and Carcinogenic Agents

Two distinct mechanisms are operative in the amplification of response to small doses of viruses of different biological types, and one of them is also involved in the amplification that occurs in response to carcinogenic agents in general.

The first mechanism is represented by a progressive pathological reaction due solely, or primarily, to multiplication and spread of the infecting agent itself, and involving little or no proliferation of the cells of the host. The viruses associated with reactions of this type, *e.g.*, those of poliomyelitis, foot-and-mouth disease, influenza, and encephalomyelitis, characteristically multiply rapidly within host cells to bring about their "explosive" death and destruction (lysis or necrosis). Virus liberated from the initially infected cells spreads by diffusion to surrounding susceptible cells and, in intact animal hosts, also by systemic circulation to distant susceptible cells. When the dose of virus is very small, only one or a few cells may be involved initially, and the reaction to the initial inoculum cannot be detected by practical means. However, as the virus continues to multiply and spread to new host cells, gross lesions (involving destroyed tissues) become recognizable or the disease becomes manifest through characteristic alterations in function, such as paralysis, or through death of the host due to the disruption of some vital function. It is these grossly detectable end-reactions that constitute the amplified response.

The second mechanism of amplification has its basis solely, or primarily, in the proliferation of host cells, whether or not the initial activating agent also multiplies along with the cells. In the carcinogenic stimuli other than viruses, the initially active agents do not multiply within the cells they invade, but bring about some "permanent alteration" that causes them henceforth to multiply at a more rapid rate than surrounding normal cells. As a result, cells of the new population "pile up" to form local tissue overgrowths or tumors. The amplification is, therefore, due solely to the proliferation of host cells. The same is true of responses to tumor-inducing viruses, (*e.g.*, various sarcomas, endotheliomas, and leukemias of chickens; Shope rabbit papilloma; mammary-gland carcinoma of mice; leukemia of mice; and kidney carcinoma of the frog) except that in this case the agent also multiplies along with the infected cells. Spread of the lesions due to the escape of virus and the infection of new cells is of minor importance, if it occurs at all, since the tumor viruses do not characteristically destroy the cells in which they multiply but rather stimulate them to increased proliferative activity. True neoplastic lesions do not retrogress, except in rare instances, but continue to proliferate until death of the animal host. On the other hand, in many extreme viral hyperplasias tumorlike overgrowths of tissue persist for varying periods before undergoing retrogressive changes and disappearing altogether. Among viruses causing lesions of this type are those of fowl pox, Shope fibroma, and the common epithelial warts which occur in various animal species. As with the true viral neoplasias, the amplification associated with these extreme hyperplasias is due primarily to the proliferation of host cells.

Still other viruses produce both cell proliferation and cell necrosis to such a degree that both processes contribute significantly to the amplification leading to grossly detectable lesions. Examples of lesions of this type are the vaccination reaction to vaccina virus, the common "fever blister" caused by herpes virus, and the "pocks" formed on chorio-allantoic membranes by various viruses (*8*).

Time Relationships of the Agent-Host Interactions Leading to Detectable Responses

One of the chief complicating factors associated with biological studies on viruses and carcinogenic agents is the extreme variation in time required for amplification of the response. Not only is there a wide variation in the over-all average and range of the time factor among agents of different types in their respective susceptible hosts but also, within any given agent-host system, there is usually a demonstrable inverse correlation between dose of agent and average time-to-response. This may be so slight—a few hours to a few days—as to be negligible for the most rapidly acting agents, such as bacteriophages and viruses of infectious diseases (in optimal experimental hosts), or it may be measured in weeks, months, and even years, as one proceeds to the less rapidly acting tumor viruses, to carcinogenic agents in general, and to the so-called "latent" viruses associated with certain neoplasias and lesions of other types. It is this enormous

variation in time that has obscured the basic similarity of the analytical methods applicable to amplified reactions in general, particularly to those obtained with various types of viruses and carcinogenic agents.

Although similar with respect to final objectives and basic principles of analysis, the specific technical operations applicable to these agents are not the same in all instances, since additional sources of variation enter in as the time-to-response becomes longer, and various factors associated with a changing age and immunological status of the host influence the course and final outcome of the agent-host interaction. Specific examples of some of the biological factors involved are given in subsequent sections. It is the purpose here to emphasize an important area in need of further biometric research and development, namely, that of the identification, characterization, and segregation of the variations associated with the different biological sources that enter the analytical picture and increase its complexity as the amplification reaction becomes more and more extended in time.

The most rapid responses do not involve appreciable changes in host status during the experimental period and can usually be handled by existing methods developed in either bacteriology or pharmacology; but in the instances thus far investigated in which the time-to-response has been more prolonged, *e.g.*, 1 to 5 or 6 weeks, as with the most rapidly acting tumor viruses, special modifications of these procedures have had to be introduced [*see* (*9*) for discussion and references]. Further prolongations in time, such as are characteristic of the response to carcinogenic hydrocarbons (*10*) and to tumor viruses of long latency, *e.g.*, those of lymphomatosis of chickens (*11–13*) and mammary-gland carcinoma of mice (*14–16*), present still other difficulties and require additional statistical controls approaching in complexity those developed in the field of epidemiology. Thus, test animals treated early in life may not develop their lesions until late in their natural lifespan, and corrections of the data for "natural" mortality become necessary (*17*, *18*). Other factors, such as intercurrent "natural" infections and transient diseases, dietary constituents, and environmental conditions, may also complicate the picture. As the amplification period becomes excessively prolonged, the biometric problem takes on the character of a combined bioassay and epidemiological methodology. This entire range of biological response data, between the established fields of bioassay methodology and epidemiological control measures, requires further investigation by biometricians and experimental biologists.

Types of Response

Regardless of differences in their specific pathological manifestations, amplified responses to viruses and carcinogenic agents fall into three distinct categories with respect to basic type and method of analysis: 1) graded (or measurable), 2) quantal, and 3) enumerative. Under appropriate conditions, responses of at least two, and sometimes all three, of these types may be observed with the same agent-host system. The

relative advantage of one type of response over another in a given instance will depend not only upon the purpose of the investigation but also upon whether or not secondary biological factors or technical difficulties interfere with the quantitative interpretation of results. Some representative examples of responses of the various types are given in the following sections.

Graded Responses

Response Time

The graded response most widely used in studies on viruses and carcinogenic agents is the time interval between treatment of a host and the appearance of a detectable reaction.[3] It is characteristic of responses to agents of these types that an appreciable period of time is required for their amplification. Response time is therefore potentially available as a criterion of agent activity in most test systems involving such agents. Whether or not it is useful for quantitative interpretations under practical conditions depends not so much on the actual magnitude of the time interval *per se* as on the amount of variation among individual host reactions in relation to the slope of the time-dose regression curve. For example, the "hosts" for bacteriophages are represented by uniform aliquots of a common bacterial suspension (or of comparable monolayers of bacteria growing on plates of solid culture media), and there is essentially no variation attributable to differences between "hosts." Response time may therefore be employed for highly accurate assays of bacteriophage activity (*20*), even though the maximum time interval reflecting the weakest response extends over only a few hours. In other systems, such as influenza virus in mice, the correlation between response and dose may extend over several days, but the relative variation among individual response times may be so great that this criterion is not very efficient as a quantitative indicator of virus activity (*21*).

The systems in which response time has proved most valuable are those involving the actions of animal tumor viruses in their natural hosts. The most rapidly acting agents of this type, such as the Rous sarcoma virus (*22*), the Shope papilloma virus (*23*), and the viruses of certain fowl leukoses (*24*), require from 1 to 5 or 6 weeks to produce reactions over their entire usable dose ranges. This is sufficient time for the hosts to develop antibodies against injected virus, or against the virus-infected cells, and there is convincing evidence in at least two tumor virus-host systems that changes in immunological status of the host occurring during the amplification period contribute in a large measure to the heterogeneity

[3] Hereinafter referred to as "response time." Responses of this type have been referred to most frequently in tumor-virus literature as "latent periods." However, because of varied uses of the term *latent* with respect to viruses, it has been suggested [Proceedings of a Symposium on Latency and Masking in Viral and Rickettsial Infections (*19*)] that a less controversial expression be adopted for designating this biological response in a biometric context. Although "reaction time" has been used in application to responses of this type in biometric literature, this same term has also been widely used in the fields of physiology and psychology for indicating a specific physiological phenomenon; it could therefore lead to confusion. Other terms that have been applied to time-lapse responses in the virus and tumor fields are "incubation period" and "induction time." These seem less appropriate for general biometric usage, however, than that suggested above, and tentatively employed herein. The author is indebted to Dr. Werner Henle for suggesting this general term.

observed among final responses (*24–26*). Thus, in titrations of the Shope papilloma virus in which various serial dilutions are inoculated at different sites on each test rabbit (*25*), the progressive prolongation of response time with decreasing dose of virus can be readily visualized on individual animals. In a few rabbits, a sudden change in the gross appearance of the lesions on a given day signals their cessation of growth and impending retrogression. From this time on, no additional papillomas appear on the sites inoculated with weaker doses of the virus, though in other animals not showing this phenomenon papillomas continue to develop progressively with descending doses over an additional period of 1 or 2 weeks. The sites that are negative at the time of the sudden change in host status, therefore, do not have sufficient time to respond to the weaker doses of virus which they have received. As a result, the quantal response to the Shope papilloma virus [*i.e.*, the tumor incidence at "infinite" time (>5 weeks)] is biologically truncated in some individuals, and is less reliable than the response time as a quantitative indicator of virus activity (*23*), unless appropriate truncation procedures are employed in interpreting the quantal data (*9*, *22*).

In the other viral neoplasia showing this phenomenon, namely, myeloblastosis of chickens (*24*, *26–28*), the change in host status is even more striking in its effect on the final results. The more susceptible hosts which develop their lesions within the first 2 or 3 weeks, after inoculation of the virus, do so with predictable regularity in relation to dose. However, the individuals of lesser susceptibility, which respond later, develop their lesions in an entirely heterogeneous fashion. Not only is the response time excessively delayed beyond what might be expected from the trend of the earlier responses but also a variable proportion of the hosts may become completely refractory and fail to show any detectable response. Because of extreme heterogeneity in the proportions that become completely refractory, the final quantal response cannot be used for quantitative purposes. Inclusion of the aberrant reactors in the quantal response data obtained with some strains of chickens introduces considerable heterogeneity and necessitates the use of extremely large numbers of test animals—600 or more (*28*)—for bioassays of reasonable accuracy [$\pm$ 0.3 $\log_{10}$ unit (*29*)]. On the other hand, the use of selected strains, and the elimination of late, and heterogeneous, responders through appropriate truncation analyses of the response-time data (*26*), results in this degree of accuracy with only about 20 to 30 test animals (*29*).

In neither of the foregoing diseases has it been possible, thus far, to develop a more ideal host system for bioassays of the tumor-producing agent. Both bioassays and studies on host reactions must therefore be carried out in the respective natural hosts.

A similar but less heterogeneous truncation has also been observed in the responses of chickens to the Rous sarcoma virus (*22*). Following the suggestion of Eckert, Beard, and Beard (*26*) with respect to myeloblastosis virus, as noted, a similar change in host status was invoked as a probable cause of the heterogeneity which complicates the response to Rous

sarcoma virus; the same truncation procedures were also successfully employed for more accurate analyses of the Rous sarcoma virus data (*22*). More recently, evidence has been obtained [4] that genetic factors are of major importance, and that lack of genetic homogeneity could account for response heterogeneity of the type observed with Rous sarcoma virus. This, of course, does not rule out a change in immunological status as the actual *modus operandi*, since the genetic influence could operate through just such a mechanism.

Although bioassays of the Rous sarcoma virus are no longer dependent on the use of the natural hosts (chickens) as test units (*31–36*), the response time as well as other graded reactions to this virus is essential for investigations on the nature of the disease in the natural hosts (*30*). The further development of biometric methods applicable to this and similar tumor-virus materials is therefore essential for future progress along quantitative biological lines. For illustrations of typical response-time data obtained with various tumor viruses *see* (*9*).

In addition to the tumor viruses, for which response time has proved to be a valuable and sometimes the only usable biological indicator, viruses of other types [*e.g.*, encephalomyelitis (*37*) and poliomyelitis (*38*) in mice and psittacosis virus in developing chick embryos (*39*)] have also yielded response-time results of equal or greater precision than quantal responses in the same hosts. A new class of viruses has recently come to light for which response time is likewise of aid as an analytical tool (*40*). This is the group of "latent" agents of the so-called adenovirus type, which normally lie "dormant" in the tissues of their natural hosts and only rarely show pathological evidence of their presence (*41–43*). It is presumed that such viruses maintain their existence by being replicated only to a slight degree as their host cells undergo normal division, and that they do not characteristically "build up" to sufficient concentrations either to damage these cells or to give any other evidence of their presence. When, however, cell-free extracts of explants of tissues harboring such viruses are transferred to susceptible "indicator strains" of cells growing in tissue culture, cytopathogenic changes and death of the indicator cells occur in relation to infecting dose of virus. The strongest doses of the adenoviruses may cause cell death within a few days, but the weakest effective doses require 20 or more days to produce their effects, even though the indicator "hosts" are tissue-culture preparations growing under optimal conditions (*40–44*). The amplification periods for such agents in tissue-culture systems therefore approach those observed for certain of the tumor viruses in their natural hosts. Since the variations between "hosts" in a tissue-culture system would be expected to be considerably less than those observed among intact animal hosts, the response times of the tissue-culture units would be expected to have a much higher inherent precision than the response times of intact animal hosts for quantitative bioassays and interpretations.

[4] Joint studies with B. R. Burmester and N. F. Waters, Regional Poultry Research Laboratory, U. S. D. A., East Lansing, Mich., and V. Groupé, Rutgers University, New Brunswick, N. J.—to be published. *See* (*30*) for a preliminary discussion.

Although bioassays are no longer necessary for the measurement of carcinogenic chemicals, quantitative biological responses to them, including response times, are essential for studies on their mode of action and on the nature of the lesions that they produce (*10*, *45*, *46*).

Other Measurable Responses

Various other graded reactions have been employed for indicating the degree of severity of virus disease or for the measurement of virus activity. Among the most commonly employed graded criteria, other than response time, are those related to the size and gross appearance of the induced lesions. Such indicators have proved most useful where the host response is not always a sharp or clear-cut reaction, *e.g.*, death-or-survival, but grades imperceptibly from such sharp reactions at the higher dose levels to barely perceptible reactions with the minimal effective doses.

An example in the category of viruses of infectious diseases is the graded series of clinical and morphological manifestations observed in mice inoculated with serial doses of the influenza viruses (*21*). Thus, very strong doses produce practically complete consolidation of both lungs, followed by precipitate death of 100 percent of the test animals within 4 to 5 days after virus inoculation. However, as decreasing doses are used the frequencies of mice showing such massive responses drop off, and a dose range is reached in which smaller total areas of the lungs are involved; also, a longer period (up to 10 days) is required for those mice to succumb. At still lower doses, only small proportionate areas of the lungs are involved and an increasing fraction of the inoculated group fails to show any detectable reaction at all. Furthermore, many of the mice developing the milder lesions recover completely after showing only slight to moderate clinical evidence of transient illness. In order to increase the quantitative precision of the biological responses in this system, Horsfall (*21*) devised a method of grading the lung consolidation at a specified time on a basis of the percentage of the total lung area showing the involvement; and, by classifying mice in broad percentage-involvement categories, he estimated the dilution which would be expected to produce just 50 percent lung consolidation, on an average. The "endpoint" dilution determined by this method is comparable to the 50 percent effective dose (ED50) in its actual usage, but it is dependent upon the observation of graded reactions within a critical dose range.

A comparable method was devised by Kenzy (*47*) for the analysis of responses to the Rous sarcoma virus, which also show graded size and morphological characteristics in relation to initiating dose [*see also* (*30*)]. Kenzy used as an "endpoint," the dilution estimated to yield tumors of a specified category (++) in 50 percent of the inoculated animals.

Although bioassay test systems of greater precision are now available for both of these viruses, there are some purposes for which the methods cited here are still among the most efficient quantitative tools that have been developed. An example is the testing of candidate chemotherapeutic agents for their ability to modify virus diseases in intact animal

hosts. Other measurable criteria that have proved of value for the latter purpose are the relative weights of the extirpated diseased organs (*48*) or tumorous lesions (*49*), at some specified constant time after virus inoculation. For additional graded reactions involving a tumor virus *see 30* and *50*.

Similar use of a series of graded morphological appearances associated with intensity of cytopathogenic effects (or "degrees of CPE") has been described by Fogh (*51*) in application to the bioassay of poliomyelitis virus in tissue culture.

Quantal Responses

The frequencies of individual hosts showing a characteristic reaction to serial doses of an agent represent the type of biological response most widely used in virus bioassays. This subject has recently been reviewed and discussed by others (*4, 52*) and is considered here, only briefly, with respect to representative examples of amplified reactions and the deviations of observed results from ideal expectancies—*see* page 141 for interpretations of quantal responses.

The quantal response (presence-or-absence of the characteristic manifestation) involves the weaker effective doses of an agent or the dose range within which some hosts respond and others do not. It has already been pointed out that the time-to-response is an important factor in any amplified reaction to viruses and carcinogenic agents, and that usually there is a significant inverse correlation between dose of agent and the time for development of a characteristic reaction. This time interval is greatest at the lower dose levels, which yield results within the critical quantal-response range. Even when the biological observation is an all-or-none phenomenon, therefore, it is essential to know the appropriate time for making such observations (*i.e.*, the point beyond which the frequencies of positive reactions no longer increase with time) or else to standardize the time of observation so that experimental truncations will not contribute to the variability of results.

In some agent-host systems, particularly where the more slowly acting agents are involved, truncations of quantal observations in time may become necessary, or may be forced technically, because of complicating biological factors. Examples of the former are the changes in host status due to such immunological factors as those cited in the discussions of response times associated with the viruses of Shope papilloma and fowl myeloblastosis, page 133.

Examples of technical truncations in time due to biological factors are seen in the limited experimental periods associated with 1) developing chick embryos and 2) certain types of tissue-culture preparations. In the first case, the chorio-allantoic membrane may not be sufficiently developed for virus inoculation before the 9th to 12th day of incubation. Examinations of the membranes usually are not satisfactory after the 19th day, and the chick hatches out on the 21st day. Only 7 to 10 days are available, therefore, for amplification of the viral lesions. This may not be

sufficient for the development of detectable responses to the weaker doses of the more slowly acting viruses, such as the tumor viruses. Even in more ideal tissue-culture systems, such as monkey-kidney cell and HeLa cell cultures used for the bioassay of various viruses, the length of the experimental period may be limited by the ability to maintain the "indicator cells" in good condition *in vitro* (*53–55*). Responses of both the quantal and enumerative types may therefore be technically truncated in time in these systems.

Another type of truncation, or of deviation from expected dose-response relationships, is that associated with "toxic" or "autointerference" properties of the agent itself, when relatively strong concentrations of it are administered to biological hosts. Factors of this sort have been described for both carcinogenic chemicals (*10*) and viruses (*44*, *56*, *57*).

Finally, when the severity or "grade" of the viral lesion varies excessively with dose and grades imperceptibly into the "negative" response, it may be necessary to truncate a graded reaction (*e.g.*, by use of a specified grade category *see* page 136) in order to have a suitable criterion for a quantal response [*see* (*58*) for a recent method of analysis of such data]. When the minimal effective doses of an agent produce their full effects within the relatively short period of a few hours to a very few days, the final amplified responses may closely follow ideal distribution curves, and the biological results may reflect, fairly faithfully, the actual size of the infecting dose. When the efficiency ratio is known (*i.e.*, the ratio of effective units to physical particles, page 141), such responses may be employed for estimating absolute quantities of virus. On the other hand, when the time-to-response is more prolonged, and complicating factors such as those mentioned enter in, the quantal responses have only relative value in quantitative work comparable to that of the graded reactions discussed in the preceding section.

Enumeration Responses

Specific responses of the enumerative type which have been employed in virus bioassays recently were reviewed and discussed by Isaacs (*52*) and Luria (*4*). They include the discrete focal reactions that occur in relation to dose of virus on such inoculated indicator surfaces as: 1) skin or corneal surfaces of animal hosts; 2) leaves of plants; 3) chorio-allantoic membranes of developing chick embryos; and 4) *in vitro* culture preparations representing monolayers of susceptible host cells. In addition, the enumeration response has proved useful as a quantitative indicator in situations in which focal systemic reactions occur throughout intact animal hosts following virus inoculations (*12*, *35*), and in specifically responding target organs, *e.g.*, the lungs of mice, following the administration of carcinogenic chemicals by various routes (*59–61*).

The achievement of focal responses that are not too dense, but yet are sufficiently numerous for accurate counts, also involves the lower effective concentrations of an agent only slightly stronger than those that are

required for satisfactory quantal responses. Similarly, the final recordings must be made at "infinite" response time or else be standardized in time so that variations due to experimental truncations will not be introduced. The same variables that influence the quantal response are effective in determining the enumeration response, and, in general, the factors described in the preceding section will cause truncations in time of the counts of focal reactions. Thus, in this instance also, limitations in the time available for bioassays are imposed by such factors as the hatching of chicks—when chorio-allantoic membranes are used—and the length of the "lifespan" of *in vitro* preparations—when the test surfaces are monolayers of cells growing in tissue culture.

Additional technical factors may complicate quantitative interpretations of the results when the test units are natural biological surfaces. For example, with certain rapidly acting viruses, secondary focal reactions may appear in the vicinity of the primary lesions as a result of local spread of the virus (*4*). This is prevented in artificial culture preparations, however, by the introduction of an agar overlay after initial infection of the indicator cell monolayers (*4*, *62*). The solidified agar prevents diffusion of liberated virus and the infection thereby of secondary sites.

Although secondary infection is not a problem with the more slowly acting tumor agents in their natural hosts, the extreme proliferative and invasive properties of cells of the neoplastic foci result in their progressive overgrowth of the intercalary areas to form continuous tumor masses. This factor complicates the enumeration response to the Shope papilloma virus, which at the weaker effective concentrations induces multiple discrete tumors when inoculated onto the scarified skin of rabbits. An additional complicating factor is that the same dose of papilloma virus may produce discrete lesions within a countable range in some rabbits but larger numbers of lesions, which rapidly become confluent, in others. The response time for a given inoculation site is determined by the first papilloma which appears. A positive quantal result is recorded for any site that develops one or more lesions.

In tissue-culture preparations, also, the numbers of focal reactions may continue to increase with time (*51*, *53*, *63*, *64*), and in some instances may become confluent if the cultures are maintained for sufficient periods (*63*, *64*). Technical truncation in time, therefore, is a standard procedure for the assay of most viruses in tissue-culture systems. In addition, the degree of sensitivity of tissue-culture units as indicators of virus activity is related in some instances to the rate at which the host cells are growing at the time of virus inoculation (*63*).

It seems likely that in many situations, particularly those involving viral agents of the more slowly developing types, factors such as those mentioned may interfere with obtaining the *true* "end titer" results. In these instances responses of the enumerative type will have relative value, only, as do the other types of responses under similar conditions.

Interpretations

Bioassays

The different principles of estimating virus activity from biological responses, indicated by the headings of the following subsections, have recently been discussed in relation to their respective spheres of applicability (*9*). A brief review will be given here of some of the facts and assumptions that have formed the bases of interpretations in the past, and a discussion of certain additional factors will also be given which may be of importance in some situations but which have not as yet received adequate consideration.

Estimation of Absolute Quantities of Virus (Virus Particle Densities)

The methods of estimating the number of virus particles from biological results are basically the same as those previously developed for determining bacterial population densities, and, in like manner, they involve both the quantal and enumerative responses (*4, 9, 52*). The theory and assumptions underlying the interpretations are the same for both types of response, and they are therefore considered together in the following discussion.

In bacterial assay systems the units of culture media are either tubes of a liquid or plates of a solid nutrient material which is optimal for growth of the specific organisms. By optimal is meant a medium that will virtually assure the establishment of an infection by a single viable bacterium. Thus, all tubes of liquid medium which receive one-or-more viable organisms will show infections due to growth of the added bacteria, and, visible colonies of bacteria will appear on the solid culture media in direct proportion to the number of viable organisms in the samples used for inoculating them. In both instances the supporting medium is a continuous phase of homogeneous, nonliving, nutrient material and there is no logical reason to consider, in the chance distribution of infections (tubes or foci), any factor other than the sampling error of the bacteria. The theory of random variation of particles in unit volumes of a suspension based upon the Poisson distribution is therefore appropriate for the estimation of bacterial population densities [*see* (*4, 9, 65*) for discussions and references to theories and methods].

The assay systems used for estimating viral particle densities, on the other hand, do not involve a physically homogeneous and continuous nutrient milieu, even when the host units are *in vitro* preparations, but suspensions or monolayers of discrete particulate units of living protoplasm (host cells). Unlike free-living organisms, viruses are dependent upon the metabolic machinery of living host cells for their replication. This fact appears not to have been taken adequately into consideration in the interpretations that have been made of responses to viruses nor in extensions to virus assays of those assumptions and theories that had previously been applied to the enumeration of bacteria.

The first strictly quantitative use of quantal and enumerative responses in virus assays were in relation to bacterial viruses or bacteriophages (*66*). Here the host cells represent bacteria taken at an optimal stage of growth from rapidly growing cultures, and maintained thereafter under optimal nutrient and environmental conditions. Under these circumstances, the host bacteria are uniformly susceptible, and viral particles in suspension induce infections with an efficiency ratio of essentially 1:1. In addition, both the quantal and enumeration responses closely follow the respective curves predicted by the Poisson distribution (*4*, *67–70*). It is obvious, therefore, that conditions can be readily established for the enumeration of bacteriophage particles by biological methods, with an accuracy approaching that achievable in estimating bacterial population densities. Certain rapidly acting animal viruses can likewise be measured in absolute units when the indicator cells are of a favorable type, such as those of developing chick embryos or of susceptible animal cells growing in tissue culture (*62*, *63*, *71*, *72*). The success with some such systems has led to the apparent assumptions that all viruses are basically similar in their interactions with host cells and that these same absolute methods should therefore be applicable to all viruses, provided that suitably susceptible indicator cells can be found or that the virus can be sufficiently "adapted" to the indicator-host cells. Certain of these assumptions were contested as generalities in 1950 by Kleczkowski (*73*), who offered experimental evidence in the plant virus field in support of his arguments. The failure of many virus-host systems (a) to closely approach the expected ratio (1:1) for the efficiency of infection by unit physical particles of the virus, or (b) to exactly follow the expected Poisson distribution, has led to some surprise and to considerable discussion of the possible reasons for the observed deviations.

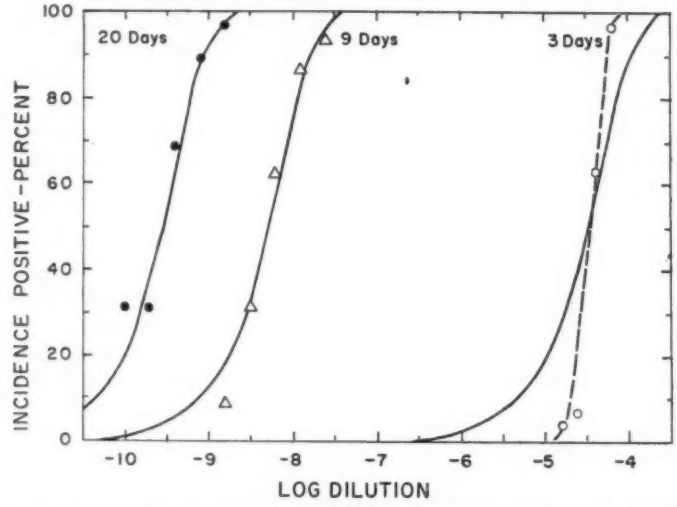
(*a*) *Deviations from the expected efficiency ratio (1:1)*

Quantal and enumeration responses.—The ratio of the number of biologically effective units to the total number of virus particles in a sample is considered the efficiency ratio. Although the ideal ratio (1:1) is seldom attained in actual assays, the approximation to it is sufficiently close in some systems [*e.g.*, bacteriophage-bacteria (*4*, *74*) and influenza virus-chick embryo (*71*, *72*)] so that for most practical purposes the titers in "infectious units" may be considered as representing physical particles of virus. In most systems, however, the ratio is significantly less than 1:1—the reported values ranging from 1:4.2 to 1:366 for viruses of infectious diseases in the most favorable indicator hosts (*52*), and from about 1:600 to about $1:8 \times 10^7$ for various tumor viruses in their respective natural hosts (*24*, *30*). Ratios as low as 1:1000 to $1:10^7$ have also been reported for assays of plant viruses (*4*, p. 54).

Isaacs (*52*) reviewed the suggested causes for lower-than-expected efficiency ratios, chief among which are " 'noninfective' particles" and " 'insusceptible' cells," and offered the additional suggestion that ". . . it is preferable to look for the reasons for failure of the cell-virus interaction

to fructify, rather than to blame one partner only." This would appear to strike closer to the heart of the problem, as it applies to viruses *in general*, than any of the single factors previously implicated, though all of them are no doubt of importance in some situations. There is considerable evidence that "failure of the cell-virus interaction to fructify," particularly within a specified experimental observation period, is the most important factor when one deals with tumor viruses, or with other viruses of the "latent" type such as the adenoviruses.

Working with an agent of the latter type in HeLa cell cultures, Pereira and Kelly (*44*) recorded quantal responses obtained at 3, 9, and 20 days, respectively, for culture preparations that had been inoculated with serial dilutions of the virus. Their results are reproduced in text-figure 1, in



TEXT-FIGURE 1.—Titer of adenovirus in HeLa cell cultures at successive times after virus inoculation; data of Pereira and Kelly (*44*). The solid curves represent Poisson "one-or-more" particle curves superposed at the observed 50 percent effective doses.

relation to theoretical Poisson curves superposed at observed ED50. At 3 days the critical dose range, within which the quantal responses decreased from 100 to 0 percent, fell between dilutions 10^{-4} and 10^{-5}. A significantly steeper slope than that expected from the Poisson distribution led them to conclude that "toxic" factors were responsible for cell death (the indicator reaction) at this strong dose level. However, both the curve observed at 9 days and that observed at 20 days followed the Poisson one-or-more particle distribution within limits of experimental error. The 50-percent-endpoint dilutions at these times were $10^{-8.3}$ and $10^{-9.6}$, respectively. The fact that the character of the dose-response curve was the same at these successive times, but varied in position with time, led Pereira and Kelly to suggest that the probability of occurrence of virus-cell interactions is a function of time. This is a particularly valuable sug-

gestion in view of similar evidence regarding the induction of tumors by carcinogenic chemicals (*60*, *75*, *76*).

Additional evidence of failure to fructify has been obtained by Manaker[5] in his studies on an adenovirus in HeLa cell cultures. The cell strain under the conditions employed had a maximal survival time of only 15 days on an average. Cultures, which had been inoculated with high dilutions (low doses) of the virus and were *still negative* at the 15-day limit, when degenerative changes began to appear in control cultures, were ground, extracted, and tested for the presence of virus in the same manner as that used in tests of original human host tissues harboring this latent virus. Thus, supernatants of centrifuged extracts were inoculated into new indicator cultures, and the latter were harvested after 6 days and extracted in like manner through three successive "blind passages." Positive results obtained after such "multiple stage amplification" of the previously "negative" cultures yielded a final dilution-endpoint two $\log_{10}$ intervals higher than that observed in the original dilution-test series. This indicates that virus and cells were involved in an interaction that failed to fructify within the 15-day interval at the higher dilutions, since free virus incubated under the same conditions in the absence of susceptible indicator cells becomes degraded (as a result of thermal inactivation) within a few hours to a very few days.

Similar conclusions can be drawn from results reported for the Rous sarcoma virus in its natural hosts. For example, Carr (*77*) studied a strain of chickens that was highly resistant to this virus and reported the appearance of a tumor at the site of inoculation in a previously "negative" chicken after a latent interval of approximately 1 year. An enduring relationship therefore existed for 1 year before fructifying.

One of the strongest arguments in favor of failure of the interaction to fructify—insofar as observable pathological reactions are concerned—is found in the natural history of viruses associated with extremely long periods of latency (*4*, *8*, *78*). Subclinical infections or "carrier states" have long been known in which viral agents (*e.g.*, those of fowl lymphomatosis, psittacosis, equine infectious anemia, etc.) multiply in and are "shed" by their natural hosts over long periods without causing any manifestation of disease. Nevertheless, their excreta and extracts of their tissues or body fluids are capable of transmitting the infection to susceptible indicator hosts.

Finally, the very existence in normal human hosts of large numbers of immunologically distinct viral agents that are capable of multiplying within and destroying indicator cells in tissue culture, but which have not as yet been linked with any known disease [*see* "Viruses in Search of Disease" (*43*), proceedings of a symposium on the subject] provides ample evidence for the coexistence of virus and cells in a state of interaction which is unfruitful insofar as an observable pathological reaction is concerned.

It is obvious that all agents listed within the general category of "viruses" do not have the same biological properties, and that they do not all follow

[5] Unpublished results of studies in progress by Dr. R. A. Manaker, National Cancer Institute, National Institutes of Health, Bethesda, Md.

the same pattern of interaction after coming into successful infecting contact with susceptible host cells. It is clear that the methods which yield fairly accurate estimates of viral particle densities in some agent-host systems may never be applicable in others, even under the best conditions. The chief experimental biological and biometric problems are, therefore, the identification of those systems to which the present bacteriological methods are not immediately applicable, and the development of quantitative methods of measurement that are appropriate for these particular systems.

It is now recognized by most virus investigators that dilution-endpoints (*e.g.*, ED50) may vary with different types of susceptible hosts as well as with different technical conditions (table 1), and that such endpoints obtained in living animal hosts cannot be used, as was previously thought possible [*see* (*24*) for review and discussion], for estimating the number of unit particles of virus in the inoculums of virus suspensions from the relationship:

$$P_{(-)} = e^{-x}$$

or

$$P_{(+)} = 1 - e^{-x}$$

where x is the average number of particles per inoculum, and $P_{(-)}$ and $P_{(+)}$ represent the probabilities of *negative* (no particles in the inoculums) and *positive* (one-or-more particles in the inoculums) quantal responses, respectively. Such variations in dilution-endpoints have been attributed chiefly to two different types of factors: 1) variations in virus particle infectivity (some particles being infectious for some hosts but not for others or under some technical conditions but not under others); and 2) variations among host cells (such as in the number of susceptible cells, the number of "points of entry" on cells, and the degree of susceptibility of cells in different types of hosts or under different conditions). Whether the source of variation has been attributed to the virus or to the host cells, the same modification of the equations given has been employed for designating the probability of successful infection under the new conditions:

$$P_{(-)} = e^{-px}$$

or

$$P_{(+)} = 1 - e^{-px}$$

where p represents the probability of either an infective particle or a susceptible cell. If both factors were of importance, an additional constant (average probability value) would have to be introduced into the equation. As indicated, it is now apparent that a third type of factor is important in some virus-host systems in determining the appearance of a detectable response, namely, the probability of fruition of a successful but subclinical virus-cell interaction *within a given interval of time*. If the latter were the only important factor (other than sampling error of the virus particles) in these virus-host systems, then p in the foregoing equations would represent this factor. If, however, one or both of the other factors were also important, two or three constants would have to be used to express the true probability of obtaining an observable response.

TABLE 1.—*Comparative titers of virus preparations under different experimentally controlled conditions*

Author(s)	Virus	Remarks	Titers under different conditions in simultaneous tests			
			Condition		Titer (ED50/ml.)	
			a	*b*	*a*	*b*
von Magnus (*79*)	Influenza A	Average of 11 determinations	Host = embryonated egg	Host = mouse	$10^{7.93}$	$10^{4.84}$
Sprunt and McDearman (*80*)	Vaccinia	Average of 3 determinations	Host = castrated male rabbit	Same, plus estrogenic hormone	$10^{9.23}$	$10^{9.01}$
		Average of 3 determinations	Host = normal male rabbit	Host = pseudopregnant female rabbit	$10^{9.42}$	$10^{9.16}$
Fulton and Armitage (*81*)	Influenza A	Average of (*a*) 2, and (*b*) 3 determinations	Host = embryonated egg	Host = isolated fragments of egg membrane	$10^{9.59}$	$10^{7.96}$
Sprunt (*82*)	Vaccinia	Comparisons at different sites on same rabbits [computations of Isaacs (*52*)]	Inoculum volume = 0.05 ml.	Inoculum volume = 1.0 ml.	$10^{8.96}$	$10^{8.13}$
Skinner and Bradish (*83*)	Vesicular stomatitis	Average of 4 determinations	Serial dilutions kept in dark for 4 hrs. at 4° C.	Serial dilutions kept in daylight for 4 hrs. at 4° C.	$10^{5.22}$	$10^{3.47}$
	New Castle disease	Average of 2 determinations	"	"	$10^{9.50}$	$10^{7.55}$
Horsfall (*21*)	Influenza A	Average of 10 determinations in mice	Response includes subtethal viral lesions	Response = mortality, only	$10^{8.60}$	$10^{7.80}$

In any event it is clear that unless the average value of p is stable, and is known, estimates of x cannot be derived from the animal responses ($P_{(-)}$ or $P_{(+)}$), but only the product px [*cf.* (*38*, *84*)] whether p derives from a single source or represents the combined probabilities deriving from several sources. When p is unknown, therefore, the product px has relative value only, and the methods of estimating virus activity from quantal and enumeration responses are identical with those discussed in subsequent sections based on other principles of potency reference.

Response time.—In an ingenious argument published in 1943, Gard (*38*) suggested a method by which the quantity px (*see* preceding section) might be estimated from response-time data, at least for some virus-host systems in which certain assumed properties of the virus-cell interaction might be valid. The method involved the same reasoning as that later employed by Meynell and Stocker (*5*, *6*) in their "hypothesis of independent action" applied to bacteria and is based upon the observed fact that the response times of mice do not continue to be correlated with dose at limiting dilutions of poliomyelitis virus, but approach a limit which is assumed to represent the average response time for the action of a single particle of the virus. Using the estimate of this limiting response time, as well as that of the slope of the time-dose regression line derived from the experimental data, Gard computed the values of px for various dilutions of virus suspension under the assumptions that: 1) p (the probability of a successful virus-cell contact, or "hit") would remain constant for the particular test system, and 2) the correlation of response time with dose at the higher dose ("multiple hit") levels is due to the increased number of virus particles (either per cell or per unit area of the infected region) and is therefore related, through the slope of the regression curve, to the actual mass of virus. The observed results gave a reasonably good fit to the expected results based on the assumptions mentioned. The estimated value of px for the undiluted virus suspension was found to be more stable, and therefore to be a better estimate of the virus titer in biologically effective units than that based on the similar px value derived from the quantal responses of the same animals. Gard concluded from these observations that the endpoint results derived from quantal responses do not distinguish between single virus particles and clumps of particles, whereas response times are independent of such differences in physical state and therefore reflect the true physical quantities of virus. If these interpretations can be substantiated through further investigations, even for a limited number of virus-host systems, a new and highly versatile approach to the study of virus-cell interactions will have been found.

(*b*) *Deviations from theoretical Poisson curves*

Deviations of observed results from dose-response curves predicted by the Poisson distribution (*see* text-fig. 2 for examples in the animal-virus and cancer fields) were first recorded in the plant-virus field. In general, the curves relating number of local lesions and dose of virus had a some-

what shallower slope than that expected from the Poisson distribution (*85–87*), though in some instances the fit to the theoretical curve was excellent. The latter was particularly true of the selected data reviewed by Lauffer and Price (*88*). Bald (*85*) considered the possibility that the shallower slope obtained in some instances could be explained by the presence of reversible aggregates of virus in undiluted virus suspensions, and the dissociation of large aggregates yielding progressively smaller ones on stepwise dilution. Modifications of the Poisson equation to take account of this factor have been discussed by both Bald (*85*) and Price (*87*). Kleczkowski (*73*), on the other hand, obtained experimental evidence for variations in susceptibility among local sites on indicator plant leaves and found that such variations followed normal distribution curves with standard deviations significantly greater than that approximated by a theoretical Poisson curve. The shallower slope of the dose-response curve was therefore attributed to biological variation rather than to aggregation of the viral particles.

In the animal virus field, deviations of observed quantal responses from theoretical Poisson curves were first emphasized by Bryan and Beard (*25*). Using data for vaccinia virus, which had been published by Parker (*89*), these authors pointed out that some of Parker's results followed a slope significantly less than that of a Poisson curve, but that all of the data published by him gave very good approximations to normal distribution curves. A second type of deviation was also noted by Bryan and Beard (*25*) in the analysis of their own data obtained with Shope papilloma virus. In this instance the higher percentage-incidence values were found to break from the trend of the lower values and to deviate significantly from both the Poisson (chart *c*, text-fig. 2) and normal distributions (*9*, *90*). This was interpreted (*25*) as resulting from a change in immunological status of some of the test rabbits as a result of the test-virus inoculations (*see* discussion on page 133). These findings led Bryan and Beard to postulate that variations among test animals constituted the most important source of variation, and to suggest that the character of the dose-response curve was determined largely by such variations rather than by the sampling error of particles *in the unit inoculums* as postulated by Parker (*89*).

This interpretation was vigorously attacked by several writers (*87*, *88*, *92*) who, however, carried the argument down to the individual host cells and the microvolumes of inoculum fluid immediately surrounding the cells. It was the random variation of one-or-more particles of virus in these microsamples that was said to determine the character of the dose-response curve, and to account for the fact that so many of the curves reported in the literature, particularly in the plant-virus field, do actually have the constant slope predicted by the Poisson equation. The latter fact, first emphasized by Lauffer and Price (*88*), is of obvious fundamental significance, and the theoretical one-or-more particle curve has come to be generally recognized as the limiting curve of error for quantal responses to particulate biological agents, such as viruses, bac-

→

TEXT-FIGURE 2.—Showing different types of deviation from theoretical "one-or-more" particle curves of quantal response data obtained with viruses and carcinogenic agents in animals.

a) Vaccinia virus in rabbits [Parker (*89*)—table 1]; 69 to 71 inoculation sites per dose group. No significant deviation.

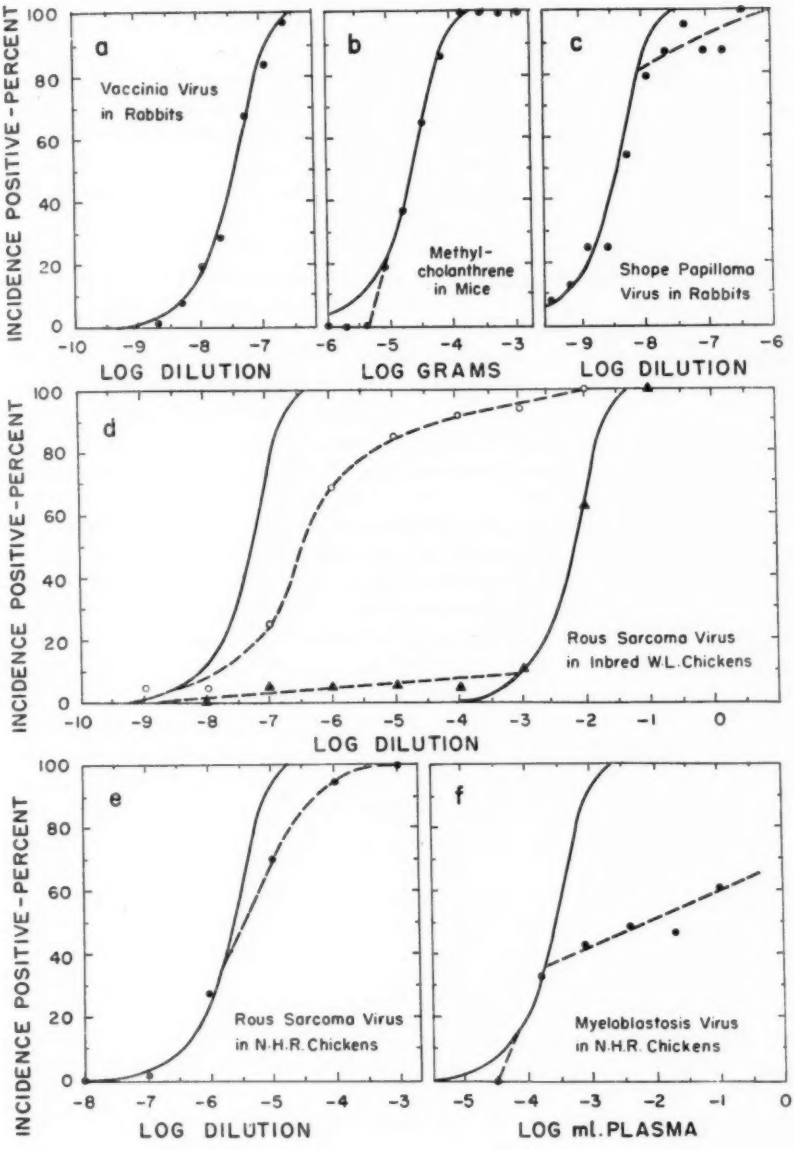
b) Methylcholanthrene in mice [Bryan and Shimkin (*10*)—table 1]; 15 to 21 mice per dose group.

c) Shope papilloma virus in rabbits [Bryan and Beard (*25*)—table 1]; 24 inoculation sites per dose group.

d) Rous sarcoma virus in inbred White Leghorn chickens (Burmester *et al.*, *see* footnote 4): *open circles* line 9, 44 to 49 chickens per dose group; *closed triangles* line 7, 19 or 20 chickens per dose group.

e) Rous sarcoma virus in commercial New Hampshire Red chickens [Bryan, Calnan, and Moloney (*91*)—table 1]; 20 (at stronger doses) to 78 (at weakest dose) chickens per dose group.

f) Myeloblastosis virus in commercial New Hampshire Red chickens [Eckert, Beard, and Beard (*28*)—table 2]; 111 to 117 chickens per dose group.


INCIDENCE POSITIVE-PERCENT
a
Vaccinia Virus
in Rabbits
LOG DILUTION
b
Methyl-
cholanthrene
in Mice
LOG GRAMS
c
Shope Papilloma
Virus in Rabbits
LOG DILUTION
d
Rous Sarcoma Virus
in Inbred W.L. Chickens
LOG DILUTION
e
Rous Sarcoma Virus
in N.H.R. Chickens
LOG DILUTION
f
Myeloblastosis Virus
in N.H.R. Chickens
LOG ml. PLASMA

teria, and other microorganisms, individual units of which are capable of initiating infection under favorable conditions (*7*, *22*, *29*, *90*, *92–99*)[6]. This concept is further substantiated by the fact that in only a few instances recorded in the literature has the observed slope for virus data been found to be steeper than that determined by the Poisson equation. In all of these instances complicating factors were reported which could account for the steeper slope, without the necessity of having to conclude that more than one particle was required to initiate an infection [*see* (*7*, *44*) for discussion of these aberrant cases].

On the other hand, many virus-host systems yield quantal responses that deviate from the one-or-more particle curve in the direction of a shallower slope. Among investigators who have dealt with this problem biometrically are Bald (*85*), Price (*87*), Kleczkowski (*73*), Matumoto, Arai, and Iwasa (*90*), Moran (*95*, *96*), Fazekas de St. Groth (*98*), Fazekas de St. Groth and Cairns (*99*), Armitage and Spicer (*84*), Meynell and Stocker (*5*), and Meynell (*6*)[6]. Factors that have been considered as being responsible for such deviations from the theoretical exponential curve are: 1) the physical state of the virus (reversible aggregation), 2) variations in infectivity among unit particles of virus (or other infectious agent), and 3) variations in susceptibility among hosts or among sites on hosts.

Of particular interest as a mathematical model for dealing with the varied types of deviations which have been observed with tumor viruses is that proposed by a number of investigators [*see* (*84*)], and further studied by Armitage and Spicer (*84*), with respect to the influence of variations in host susceptibility on the nature of the dose-response curve; *i.e.*,

$$P_{(-)} = e^{-pz}$$

(*see* page 144).

Armitage and Spicer regarded the proportion p as the probability that a particle chosen at random would be infective, and treated it as a variable dependent upon the *susceptibility* of the host. They assumed different distributions of p and showed that significant deviations from the exponential curve would result from variations in p only at the higher incidence levels, unless the value of average p was very small or the variations in host susceptibility were very heterogeneous. Although the results of figures 1 and 2 published by Armitage and Spicer were in terms of the loglog transformation, the curvatures of the dose-response curves at the higher positive response (lower negative response) levels are analogous to the "truncations" in the probit plot of certain tumor-virus data (*9*, *22*). Further investigations by Armitage[7] have revealed that many of the bizarre dose-response curves obtained with viruses (text-fig. 2) can be closely approximated by appropriate assumptions regarding the level of magnitude and the nature of the distribution of the p values substituted in the above equation. Of particular interest was the duplication of the

[6] Reference added in proof: IWASZKIEWIEZ, K., and NEYMAN, J.: Counting virulent bacteria and particles of virus. Acta biol. exper. 6: 101–142, 1931.

[7] Personal communication and oral discussion given in a lecture at Fort Detrick, Frederick, Md., January 9, 1958.

opposite "truncations" observed with two chicken lines of different genetic susceptibility to the Rous sarcoma virus (chart *d*, text-fig. 2) by assuming a "two point distribution" with average p value taken from the published dose-response data. Also it was found that appropriately assumed magnitudes and distributions of p could result in dose-response curves of a shallower slope than the exponential over most of their courses. Such curves were approximately linear in terms of the probit-log dose plot within the significant quantal-response range—a result frequently obtained with tumor viruses (*9*, *22*, *24*).

In all the biometric studies published thus far, the proportion p has been taken to represent either "infectivity" of viral particles or "susceptibility" of hosts to infection, and the probability of susceptible cells becoming infected, but not showing "fruition" (page 141), has not been taken into consideration. The latter factor might be considered of academic importance, only, were it not for the fact that certain crucial biological interpretations have been based on the mathematical nature of the dose-response results, under the assumption that the conditions for grossly visible responses are the same for viral as for bacterial test systems. For example, although it appears firmly established that one physical particle of virus is capable of initiating an infection (under favorable conditions), it does not necessarily follow that isolated lesions or positive quantal reactions at *apparent* limiting dilutions have been generated entirely by single particles of virus, an assumption frequently made. An illustration is apparent in the results of Pereira and Kelly (*44*), discussed on page 142, in which Poisson one-or-more particle curves were obtained at different levels of dilution after successive intervals of time. Also, as illustrated by Manaker's studies, discussed on page 143, true limiting dilutions for successful infection were not reached during the "survival time" of indicator-host cells in tissue culture. Several, or even many, successfully infected cells might have been present in the cultures which did become grossly "positive" at the *apparent* limiting dilutions. Similarly, local lesions, or pocks, recovered for the purpose of "cloning" virus strains might actually contain many successfully infected cells—the fruition of only one of which was necessary to start the chain reaction leading to a grossly visible "colony." That such quiescent infections might become activated by the developing reactions in closely adjacent cells (and thus contribute to the viral population of an isolated "colony") seems probable, for certain viruses at least, from the "autocatalytic" nature of the dose-response-time curve which has been discussed in a similar connection by Gard (*38*).

In the responses to viruses, therefore, the presence or absence of particles in microvolumes of inoculum in contact with cells is not the only random variable that can account for an exponential curve of the one-or-more particle type. The fruition of one-or-more successfully infected cells within a given interval of time would also lead to a Poisson distribution. Since the number of successfully infected cells in a microarea (or the number of viral particles per cell) would directly influence the probability of one-or-

more cells undergoing fruition within a given interval of time, the observed responses would be directly related to dose of virus. The general assumption that adherence to the Poisson distribution "proves" that local lesions (or positive reactions) obtained with apparently limiting dilutions are generated by single particles of virus is not necessarily valid for all virus-host systems.

Another crucial interpretation involves the use of biological units (*see* succeeding section) as a basis of biological standardization. In certain types of viruses, at least, the possibility exists that failure to recognize a residuum of successful but *inapparent* cellular infections in the indicator systems could lead to serious errors in the estimates of absolute quantities of virus.

Potency Estimates in Biological Units

A biological unit may be defined as the quantity of agent (not necessarily known in absolute amount) required to produce some specified reference response under a standardized set of test conditions, including the indicator-host population. The biological units most commonly employed in work with viruses are: 1) the dose giving the 50 percent quantal response (ED50), and 2) the "pock-forming unit (PFU)," which is the dose required on an average to produce 1 local lesion per unit volume of inoculum on an indicator surface.

In a few favorable virus-indicator systems the ratio of biological units to physical particles approaches unity within a factor of 3 or less (*4, 63, 71, 72, 74*) and, for all practical purposes, biological units may be taken to represent known physical quantities of virus. Under these circumstances, kinetic studies on virus-cell interactions (*e.g.*, involving a number of ED50's or PFU's in relation to a known number of host cells) have a definite quantitative meaning in terms of approximately known physical quantities of virus. At the other extreme, several virus-host systems have been recognized in which the population variability of the reference response is too great (*100–104*), and/or the ratio of biological units to physical particles of virus is too low [*24, 30*; *see also 4*, page 54] for interpretations, in terms of biological units, to have any tangible meaning with respect to actual physical quantities of virus. Under these circumstances, biological units do not represent satisfactory reference criteria for strictly quantitative evaluations, and the principle discussed in the succeeding section must be employed for analytical bioassays.

The chief problems of interest for further bioexperimental and biometric consideration are: 1) interpretations of results falling between the foregoing extreme types, and 2) the determination of conditions under which biological units have a valid quantitative meaning. For example, if it is assumed that a low efficiency ratio represents simply the fact that only a small portion of the total virus-particle population is infectious, and that the efficiency of infection by particles of this particular class is relatively high, then biological units may be assumed to represent, approximately, actual numbers of *infectious* virus particles. Interpretations in biological

units would therefore have a valid quantitative meaning with respect to particles possessing the property of infectiousness. This, in fact, appears to be the basic assumption underlying the widespread use, at the present time, of biological units in studies on viruses, even when the actual magnitudes of the efficiency ratios are unknown.

The foregoing may not always be a valid assumption, however, since, as discussed on page 143, certain types of viruses may induce subclinical infections that fail to manifest themselves within the experimental observation period or under the conditions of the tests. Low efficiency ratios may therefore be related to conditions influencing the fruition of virus-cell interactions rather than to proportions of physical particles possessing the ability to infect. Under these circumstances, biological units would have no real quantitative meaning with respect to absolute virus amount when efficiency ratios are unknown.

Potency Estimates in Relation to Standard Preparations of Agents

When absolute estimates of viral particle densities are not feasible, and when there is significant variation among host lots in average susceptibility to a virus, the only means of quantitative bioassay is by the analytical method now generally employed for the biological standardization of drugs. This method is so well known and so highly developed, as to require no further discussion. *See 65* and *105* for historical reviews, and *9*, *24*, *38*, *86*, *87*, and *103* for discussions of some applications to viruses.

The chief problems in the application of this method to viruses reside in 1) the preparation and maintenance of standard samples of virus, and 2) the analysis and interpretation of dose-response data obtained with viruses. The first type of problem is beyond the scope of the present discussion (*cf. 38*, *100*, *101*, *103*, *104*, *106*). The second involves the response characteristics, and related analytical problems, considered in some detail in earlier sections of this report.

Host Reactions

Biometric studies pertaining to host reactions *per se* have centered around two general objectives. The first involves the use of biometric methods, as working tools for the mathematical expression, and summarization of empirical findings relative to host factors which influence agent-host interaction. The second may be looked upon as "theoretical biology." It has as its purpose the testing of various theoretical mathematical models for their ability to describe and predict observed data, and thus to reveal basic knowledge concerning the nature of biological phenomena as well as fundamental laws governing agent-host interactions.

The use of biometric tools for empirical studies on host reactions requires little additional comment since the specific problems involved in analyses of such data are the same as those employed in summarizing host-response data for the purpose of bioassay [*cf.* (*30*, *50*)]. Of partic-

ular interest for further biometric and theoretical research are the detailed studies that have been made of dose-response relationships in investigations on the reactions of a wide variety of hosts to controlled doses of various viruses and carcinogenic agents, many of which have been cited in these pages. For the most part, such studies have been made primarily for the purpose of determining the appropriate metameters and biometric procedures to be used in routine bioassays. Nevertheless, they constitute a valuable backlog of empirical data (on the magnitudes and distributions of host reactions) which may be used for further investigations along both methodological and theoretical lines.

Reference has already been made to some theoretical studies directed toward biometric methodology (page 150). Theoretical considerations have also been directed toward the elucidation of basic biological phenomena in relation to controlled doses of causative agents in both the virus (*38*, *107*) and cancer (*75*, *76*, *108–114*) fields. A detailed review of such investigations is beyond the scope of the present undertaking. It is hoped, however, that the references cited herein may serve as a guide to interested mathematicians, and that the biological considerations of preceding sections may provide some insight into the nature of some of the problems that must be dealt with in the further advancement of quantitative and theoretical biology in the virus and cancer fields.

Since the observable responses to viruses and carcinogenic agents represent "amplified" reactions dependent upon the multiplication of viral and/or cellular biological elements, it seems probable that the final solutions to certain theoretical problems, as well as an understanding of certain basic biological phenomena associated with complex agent-host interactions, will require the use of stochastic mathematical models along the lines pioneered by Hoffman, Metropolis, and Gardiner (*115*, *116*).[8]

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Induction of Tumors in Rats by Tissue-Culture Preparations of SE Polyoma Virus [1]

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Summary

Sixty-five newborn Sprague-Dawley rats were inoculated with SE polyoma virus that had been propagated in mouse-embryo tissue cultures. Eighteen of the rats developed renal sarcomas and 7, subcutaneous tumors. A tissue mince of renal sarcoma and spleen from a rat with an induced kidney tumor was used as an initial inoculum for a series of mouse-embryo tissue cultures. The culture fluid from the fourth serial passage induced visceral tumors in hamsters like those produced by previous tissue-culture lines of the SE polyoma virus. The fluid from this culture after 12 serial passages again induced renal sarcomas in rats. The rat is the third mammalian species to develop significant numbers of tumors after receiving, in the newborn period, SE polyoma virus grown in tissue culture.—**J. Nat. Cancer Inst. 22: 161–171, 1959.**

Recent investigations (*1–5*) indicate that tissue-culture preparations inoculated with a replicating agent, the SE polyoma virus, induce tumors in (C3Hf × AKR)F_1 hybrid mice, randomly bred Swiss mice, and hamsters. Properties which indicate that the replicating agent is a virus are being reported in current publications (*6–8*). The present report describes the method of inducing neoplasms in randomly bred Sprague-Dawley rats and the recovery of the virus from mouse-embryo tissue cultures inoculated with an extract of tumor tissue from a rat that had received the SE polyoma virus when newborn.

Materials and Methods

The detailed history of the 2 virus cultures used in these studies, lines 3469 and 3919, was given in a previous publication (*3*). Both lines are related through their common origin from tissues of a single AKR mouse that developed leukemia spontaneously. Tissues from the AKR mouse, which were infiltrated with leukemic cells, were used as an inoculum for a

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[2] Division of Biologics Standards.
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[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

monkey-kidney tissue-culture preparation designated 24424. The decanted fluid from tissue-culture line 24424, passage 2, induced salivary-gland tumors in (C3Hf × AKR)F_1 hybrid mice. An extract of the resultant salivary-gland tumors of 1 mouse was used to inoculate the first of a series of monkey-kidney and mouse-embryo tissue cultures. The decanted fluids derived from these cultures were capable of inducing multiple tumors in mice and hamsters and were designated 3469. Pooled tissues from 2 (C3Hf × AKR)F_1 hybrid mice that had received fluid from the fourth passage of tissue-culture line 3469 served as inoculum for a third series of tissue cultures designated 3672. A fourth series, 3919, was initiated by inoculating mouse-embryo tissue cultures with the extract of a parotid-gland tumor induced in a (C3Hf × AKR)F_1 hybrid mouse with fluid from the third culture passage of line 3672.

The early passages of 3469 were made in monkey-kidney tissue cultures; later passages, including the ones used in this study, were from Swiss mouse-embryo tissue cultures. Passages were made by transferring 2 ml. of fluid from a culture that had been incubated at 36° C. for 14 days or longer to a 2-ounce prescription bottle containing a monolayer of mouse-embryo cells and 8 ml. of medium. The cultures were nourished with synthetic medium 199 (*9*) and 1 percent calf serum. Nutrient fluids were replaced at weekly intervals, but only fluids removed after 2 or more weeks were transferred to new cultures or used for animal inoculation.

Five litters of rats were inoculated with culture-grown virus preparations 3469 and 3919 (table 1). In experiment 1 a single litter received the decanted fluid from the thirteenth passage of tissue-culture-grown virus preparation 3469, and in experiment 2 a litter received decanted fluid from the nineteenth passage. In experiment 3 a litter of rats was divided; 6 of the litter received the supernatant fluid of centrifuged virus preparation 3469, culture-passage 20, and 8 received sediment from the same virus preparation. In experiment 4, 1 litter received the supernatant fluid from centrifuged virus preparation 3919, culture-passage 10, and a second litter received the sediment.

Separation of the fractions in experiments 3 and 4 was carried out by first removing cellular debris in the tissue-culture fluid by centrifugation in an angle centrifuge at 4000 rpm for 20 minutes. The decanted fluid was measured and normal rabbit serum was added to a concentration of 1 percent. The mixture was then centrifuged in a #40 rotor in a Spinco high-speed centrifuge, model L, at 85,780 to 142,900 × g for 3 hours. The top milliliter of fluid in each 10 ml. cup served as the supernatant inoculums in experiments 3a and 4a. The pellet on the bottom of each cup was suspended in 1 ml. of Earle's balanced salt solution (*10*) and served as the sediment inoculums in experiments 3b and 4b.

A virus tissue-culture line, 632, was initiated by inoculating a 5-day-old mouse-embryo tissue culture with the minced spleen and tumor tissue from a rat with an induced renal tumor. The culture was maintained as previously described, except that horse serum rather than calf serum was used in the maintenance medium. Fluid from this culture was passed

serially to new cultures as described earlier (*1*, *3*). The fourth culture passage of line 632 was used to inoculate a litter of seven 3-day-old hamsters, and the twelfth passage was given to a litter of 11 newborn rats.

Tissue-culture-grown virus, in 0.25 ml. amounts, was injected into the subcutaneous tissue of the back of all rats when they were less than 24 hours old. The hamsters received 0.2 ml. of a virus tissue-culture preparation at the same site. The animals were raised in the Animal Production Section of the National Institutes of Health. The rats were pen-bred descendants of the Sprague-Dawley line, and the hamsters, *Cricetus auratus*, were randomly bred. The animals were housed in metal, shoebox cages and given unrestricted quantities of Purina laboratory chow and water. All inoculated animals were allowed to remain with their mothers until at least 21 days of age. They were observed daily for death or signs of disease. Moribund animals were killed. Tumors in either rats or hamsters, unless they occurred subcutaneously, were not noted until the time of autopsy. Selected tissues of rats with macroscopic tumors were fixed in 10 percent formalin, embedded, sectioned, and stained with hematoxylin and eosin.

Results

Induction of Tumors With Tissue-Culture Passages of Extracts of Mouse Tumors

The results of inoculating newborn Sprague-Dawley rats with virus tissue-culture preparations are given in table 1. The first four experiments are represented by 54 rats inoculated with tissue-culture passage of lines initiated with extracts of mouse tumors. Eight of the 54 animals died, and these decomposed to such an extent that the animals were unsuitable for adequate *post-mortem* examination. Of the effective total of 46 rats, 4 developed subcutaneous tumors and 12 developed renal tumors. In the 2 experiments designed to concentrate the active principle, 1 of 15 rats inoculated with supernatant fluid from material centrifuged at 85,780 to 142,900 $\times$ g developed a renal tumor, while the sediment induced 2 subcutaneous tumors and 9 renal tumors in the 21 animals inoculated. It is important to note that sediment from tissue-culture line 3919, passage 10, was the most potent preparation used, causing 9 of 13 rats to develop renal tumors and accounting for half of all the renal tumors induced. As yet we have no complete explanation for the apparent variations in the potency of a given virus culture preparation. It is obvious that this particular passage of 3919 was more potent than the 3 passages of 3469 which were tested, and that centrifugation is capable of concentrating the tumor-inducing factor.

Induction of Tumors With Fluid From Tissue Cultures Incubated With an Extract of an Induced Rat Tumor

Table 2 shows the grossly observed tumors noted in a litter of hamsters that had been inoculated with the fourth passage of culture line 632,

TABLE 1.—*Response of Sprague-Dawley rats inoculated at birth with mouse-embryo tissue-culture preparations of the SE polyoma virus*

Expt. No.	Virus line*	Fraction of virus preparation used	Rats inoculated	Rats lost	Effective total	Rats with subcutaneous tumors	Rats with renal sarcomas	Rats surviving without tumors	
								Number	Days
1	3469–13	Decanted fluid	6	1	5	1	2	2	469
2	3469–19	Decanted fluid	12	4	8	1	0	7	385
3a	3469–20	Supernatant fluid	6	0	6	0	1	5	354
3b	3469–20	Sediment	8	1	7	1	0	6	354
4a	3919–10	Supernatant fluid	9	0	9	0	0	9	350
4b	3919–10	Sediment	13	2	11	1	9†	1	350
5	632–12	Decanted fluid	11	0	11	3	6	5	126

*The last two numbers designate the particular tissue-culture passage used as the inoculum.
† Tissue from renal sarcoma and spleen of one rat used as inoculum to initiate virus-culture line 632.

which had been recovered from a rat with an induced renal sarcoma. The character and distribution of the induced tumors were similar to the tumors induced in hamsters by SE polyoma virus-culture fluid (*3*). The results for 11 newborn rats that received virus preparation 632 are shown in table 1, experiment 5. Renal sarcomas occurred in 2 rats that died at 56 days of age, in 3 that died within a 2½-month period, and in 1 that died at 4½ months. The remaining 5 rats under observation for 5 months show no sign of disease. Five of the 6 rats with renal sarcomas had bilateral tumors, and 1 showed an early hemangioma of the liver that might have represented a metastasis. Three of the animals also had subcutaneous tumors.

TABLE 2.—*Distribution of gross neoplasms in 7 hamsters receiving 4th passage tissue-culture line of 632 at 3 days of age*

Hamster number	Age at death (*days*)	Sites of tumors:*				
		Lung	Liver	Heart	Kidney	Gastro-intestinal tract
1	Less than 4					
2	18	+	+			
3	28	+	+	+		+
4	28	+	+	+		
5	34		+			
6	35	+	+	+	+	
7	39	+		+		

*Grossly noted tumors in lungs and livers consisted of multiple blood-filled loculi; tumors in the other organs were solid circumscribed masses which occurred on the serosal surface of the gastrointestinal tract and heart, and within the myocardium and renal pyramids. All tumors were macroscopically similar to those described previously by Eddy *et al.* (*3*).

Pathology of the Induced Tumors

Selected tissues from 21 rats were studied histologically. The tissues generally included were the heart, lungs, salivary glands, liver, spleen, and kidneys. Four rats each had single subcutaneous tumors. On histologic examination 2 of these tumors had admixtures of stromal and glandular elements typical of the murine fibroadenoma of the breast. The remaining 2 subcutaneous tumors showed evidence of invasive growth and marked anaplasia of connective-tissue cells; 1 of these contained areas of neoplastic fat cells as well as fibrosarcomatous elements. Eighteen rats had tumors originating in the kidneys; in 10 animals the renal tumors were bilateral. The renal tumors were sufficiently alike histologically to be considered as a group. The site of origin of the smaller tumors was near the corticomedullary junction. Growth occurred through extension to the renal papillae and into the pelves of the kidneys. In most cases, even where there was retroperitoneal extension, a thin strip of subcapsular cortex survived. Grossly, the tumors were red due to abundant vascularity. Retroperitoneal hemorrhage from the tumors caused some deaths. In 2 of the animals direct growth of the renal tumor had involved much of the retroperitoneal space and had penetrated the peritoneum where

contiguous growth involved the serosa of the omentum, mesentary, and gastrointestinal tract (fig. 1). The renal tumors of these 2 animals represented the largest primary growths in the group, each measuring over 5 cm. in greatest diameter.

Histologically, all the renal tumors were composed of both solid cell masses and cavernous blood-filled spaces that were lined by tumor cells. The cavernous vascular pattern predominated. The larger spaces were filled with organized thrombi and were lined by multicellular layers of neoplastic cuboidal cells supported by a scant fibrous stroma (fig. 2). Papillary cords of such tissue sometimes separated the vascular spaces into incomplete loculi. At the margins of these areas various neoplastic cell types could be noted. Large fusiform cells were prominent and these areas contained bizarre, sometimes multinucleated, giant cells similar to cells seen in myosarcomas (fig. 3). Myofibrils were apparent in some tumors but striated muscle fibers could not be identified. In other areas, elongated spindle cells with blunt cigar-shaped nuclei formed whorled sheaves and palisading arrangements separated by wavy bundles of collagen—patterns associated with the growth of smooth muscle tumors. Frequently within the same tumor, large areas of closely packed polymorphous cells with large ovoid nuclei, prominent nucleoli, and scant cytoplasm could be found. Such areas frequently contained blood-filled clefts and spaces lined only by tumor cells (fig. 4). In other tumors thin sheets of similar, rapidly growing cells were surrounded by a dense eosinophilic, osteoid-like ground substance which contained areas of mineralization. Mitotic activity in the tumors varied with the degree of anaplasia. The less-differentiated areas showed as many as 3 to 4 mitotic figures per high-power field, while areas of the tumors with well-defined vascular or muscular cells rarely contained mitotic figures.

Three of the 18 rats with renal sarcomas had tumor deposits within pulmonary veins which showed extension into surrounding pulmonary tissue. The pulmonary metastases mimicked the primary renal sarcomas even to the occasional formation of cavernous hemangiomatous spaces (fig. 5). We have tentatively designated the renal tumors as angiosarcomas, but it should be emphasized that the malignant potential of these tumors has not been thoroughly evaluated. The vascular origin is based on the more clearly defined neoplastic vascular elements of the primary tumors and of their metastases; however, not all the cellular changes observed fit into a simple pattern of vascular neoplasia. Several of the tumors showed apparent osteoid formation and calcification. It is quite possible that the vascular pattern represents only one of several lines of neoplastic growth by totipotential cells of the renal blastema. None of the tumors contained identifiable epithelial elements nor did they show any close resemblance to the embryonal adenosarcomas of human kidneys.

Discussion

The significance of the subcutaneous tumors that developed in 7 of the 65 rats is uncertain because such tumors occur occasionally as spontaneous

tumors in our colony. There is little doubt about the significance of the renal sarcomas that developed in 18 of the 65 rats inoculated—an over-all incidence of 28 percent. The experience of Davis *et al.* (*11*), with 150 aged female Sprague-Dawley rats, and Simms *et al.* (*12*), with over 2,000 Sprague-Dawley rats allowed to live a normal lifespan, indicates, even better than our limited experience, that the incidence of spontaneous renal sarcomas is probably much less than 1 percent. It is apparent that the response of the rat to tumor induction by our technique is not as striking as it is in either the hamster or the mouse. Unequivocally induced primary tumors of the rat were all sarcomas that arose in the kidney, while other tissue-culture passages of lines 3919 and 3469 were capable of inducing primary epithelial and mesenchymal tumors in many sites in mice (*5*), and primary mesenchymal tumors in the lungs, liver, kidneys, heart, serosal tissues, and subcutaneous tissues of hamsters (*3*).

There is considerable variation in the response of the hamster and the mouse to various culture passages of the virus. These variations may depend on both the potency of the preparation and the susceptibility of the animal inoculated. Recently, we have shown that the incidence of tumors is dependent on the dosage of the inoculum (*5*). It is therefore significant that where sedimentation was attempted by high-speed centrifugation, the greatest number of renal tumors was induced by the fraction containing the heavier sedimented particles. This technique may serve as an effective means of concentrating and purifying the agent responsible for tumor induction.

A single attempt to recover a tumor-inducing factor from serially passed tissue cultures inoculated with material from an induced tumor in a rat was successful. This supports our thesis that a replicating agent, the SE polyoma virus, or the product of the interaction of this virus and tissue-culture cells is responsible for the tumors induced.

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PLATE 19

FIGURE 1.—Renal sarcoma in the kidney of a rat with extension of the tumor to peritoneal surfaces of abdominal viscera.

FIGURE 2.—Rat renal sarcoma showing hemangiomatous pattern of growth. Hematoxylin and eosin. × 210

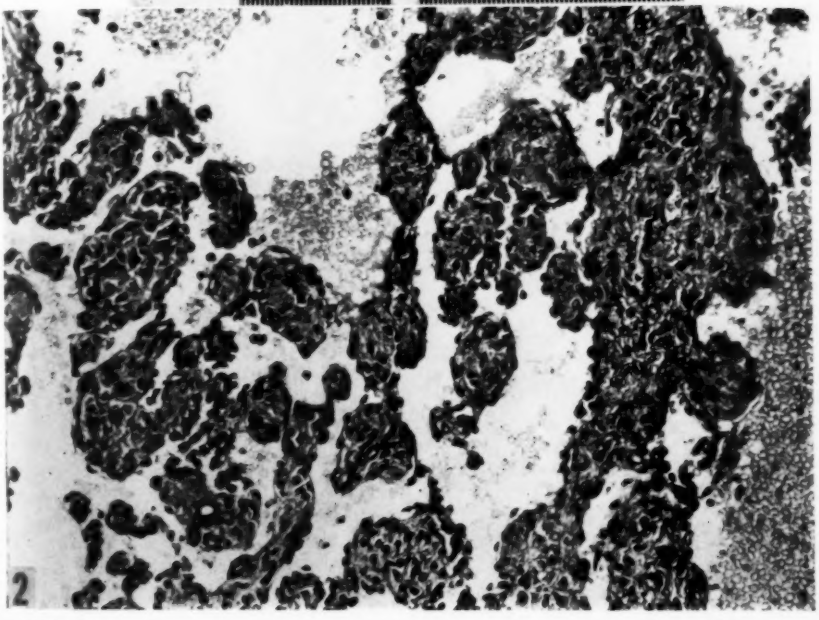

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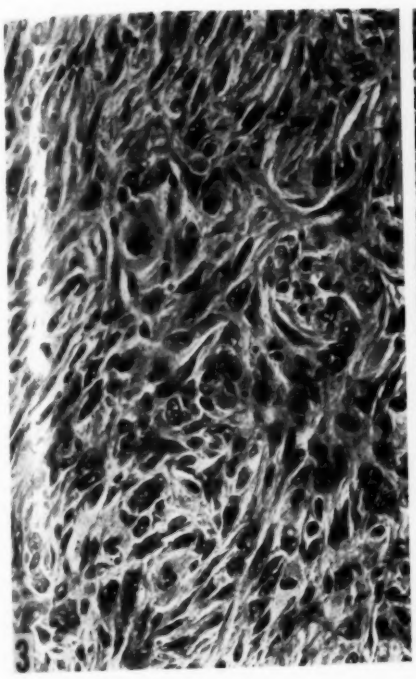
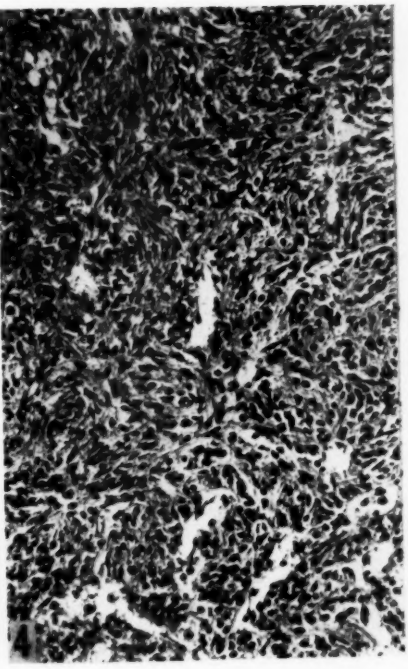
PLATE 20

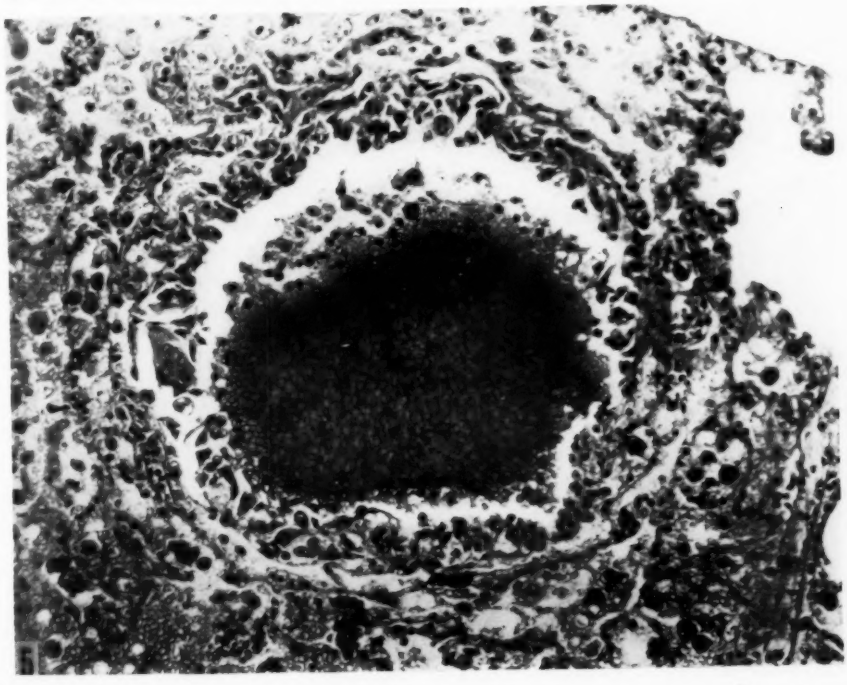
FIGURE 3.—Rat renal sarcoma showing spindle cells with fasciculated pattern and multinucleated tumor cells with abnormal mitotic figures. Hematoxylin and eosin. × 260

FIGURE 4.—Rat renal sarcoma showing capillary vasculature and fasciculated pattern. Hematoxylin and eosin. × 210

FIGURE 5.—Rat lung showing intravascular metastasis from renal sarcoma. Hematoxylin and eosin. × 210







Smoking and Lung Cancer: Recent Evidence and a Discussion of Some Questions [1]

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Summary

This report reviews some of the more recent epidemiologic and experimental findings on the relationship of tobacco smoking to lung cancer, and discusses some criticisms directed against the conclusion that tobacco smoking, especially cigarettes, has a causal role in the increase in bronchogenic carcinoma. The magnitude of the excess lung-cancer risk among cigarette smokers is so great that the results can not be interpreted as arising from an indirect association of cigarette smoking with some other agent or characteristic, since this hypothetical agent would have to be at least as strongly associated with lung cancer as cigarette use; no such agent has been found or suggested. The consistency of all the epidemiologic and experimental evidence also supports the conclusion of a causal relationship with cigarette smoking, while there are serious inconsistencies in reconciling the evidence with other hypotheses which have been advanced. Unquestionably there are areas where more research is necessary, and, of course, no single cause accounts for all lung cancer. The information already available, however, is sufficient for planning and activating public health measures.—**J. Nat. Cancer Inst. 22:173–203, 1959.**

In 1957 a Study Group (*75*), appointed by the National Cancer Institute, the National Heart Institute, the American Cancer Society, and the American Heart Association, examined the scientific evidence on the effects of tobacco smoking on health and arrived at the following conclusion:

> "The sum total of scientific evidence establishes beyond reasonable doubt that cigarette smoking is a causative factor in the rapidly increasing incidence of human epidermoid carcinoma of the lung."

Concurrently, a report from the Medical Research Council (*57*) of Great Britain appeared which also drew the inference of a causal relation-

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ship between smoking and lung cancer from the statistical, clinical, and laboratory evidence available by midyear 1957.

The consideration of the accumulated scientific evidence has led to the acceptance of a similar viewpoint by responsible public health officials in Great Britain, the Netherlands, Norway, and the United States. This concensus of scientific and public health opinion does not mean that all problems regarding smoking and lung cancer have now been solved or that valid questions and reservations about some aspects of the subject do not remain. An excellent collection of primary references and opinions expressing both "sides" of the question was issued by a committee of the House of Representatives (*42*) which sought to examine the claims of filter-tip cigarette advertisements.

The general acceptance of the cigarette–lung-cancer relationship has not decreased research interest but has accelerated research in this and in such related fields as respiratory physiology and environmental carcinogens, and on the effect of tobacco smoke in a wide range of physiological and pathological reactions.

The result is that considerably more information has been published or has become available through other media. Included in the recent scientific evidence are the following:

1) Additional retrospective studies (*68*, *69*, *73*) on men with lung cancer and on matched controls have appeared. All show an association between cigarette smoking and epidermoid-undifferentiated lung cancer.

2) Additional retrospective studies on women (*34*, *73*) also show the association.

3) The first results of a third large prospective study (*20*), which included 200,000 United States veterans who were observed for 30 months, duplicate closely the reported findings of the Hammond-Horn (*38*) and the Doll-Hill (*18*) studies.

4) Analyses by Kreyberg and others (*19*, *46*) substantiate that, epidemiologically, primary lung cancer must be divided into epidermoid-undifferentiated and adenocarcinoma. The latter is much less related to smoking and, so far as is known at present, to other carcinogenic inhalants.

5) Additional findings have become available on the impingement of tobacco-smoke particles in the bronchi of animals, ciliary paralysis, and penetration of unidentified fluorescent materials into the bronchial cells (*40*, *41*, *45*).

6) Additional data have been published (*2*, *12*) on the more frequent occurrence of hyperplastic and metaplastic changes in the lungs of smokers as compared with the lungs of nonsmokers. Hyperplastic and metaplastic changes have been produced in bronchi of dogs exposed to direct contact with tobacco "tars" (*62*) and in bronchi of mice exposed to tobacco smoke (*48*).

7) Additional confirmations have been obtained on the induction of cancer of the skin in mice painted with tobacco-smoke condensates (*7*, *23*, *28*, *61*, *63*).

8) Progress continues on the isolation and identification of chemical

constituents in tobacco smoke, including compounds of the carcinogenic polycyclic type (*53, 61, 77, 84, 85*).

The growing and consistent body of evidence has had no noticeable effect upon the viewpoint of a small but important group of individuals who would deny a causal role of cigarette smoking in cancer of the lung. Among these critics are Little (*52*) and Hartnett (*39*), spokesmen for the American tobacco industry. Berkson (*3, 4*) has been critical of many aspects of the statistical studies, and his reservations are, in part, also evident in papers by Neyman (*60*) and Arkin (*1*). More general objections by Fisher (*25, 26*), Greene (*31*), Hueper (*43*), Macdonald (*54*). Rigdon (*64*), and Rosenblatt (*67*) have been published.

We have reviewed the criticisms that have been made regarding the cigarette–lung-cancer relationship in the light of new evidence. In this review we have several objectives: *a*) to point out recorded facts that directly answer some of the criticisms; *b*) to define more precisely some inadequacies of information, with the hope that this will lead to further research. The particular references we have used were selected because in our opinion the criticism was well stated; it is not our intention to reply to any specific publication or to any specific critic. Our view is that all valid questions should be answered. However, some questions may not be relevant, or there may be no information presently available for an answer. In the latter case, we believe that a distinction should be made between data that are unavailable and data that have been found to be contradictory.

For convenience, we have divided the criticisms and answers into five major topics, as follows: (I) Mortality and population data; (II) Retrospective and prospective studies; (III) Studies on pathogenesis; (IV) Other laboratory investigations; and (V) Interpretation.

I. Mortality and Population Data

The rising death rate from lung cancer in all countries that have sufficiently detailed mortality statistics is the most striking neoplastic phenomenon of this century. That this increase is a fact and not a spurious result of statistical classification is now commonly accepted. An entirely contrary view is held by only a few persons (*64*), though there are dissenting opinions (*29, 43*) regarding the extent and time relationship of this recorded increase.

Obviously, the case for the etiologic role of cigarette smoking would be seriously compromised if it could be demonstrated that the lung-cancer rate over the past half century had been stationary, particularly after 1920 when much of the rise in cigarette consumption, instead of other forms of tobacco, occurred (*59*).

In a recent review, Rigdon and Kirchoff (*65*) document that primary lung cancer was first recognized as an entity during the early part of the 19th century, and that its occurrence has increased steadily since then, as manifested by the recorded relative frequency with which it was recognized in the clinic and at necropsy. This is undoubtedly correct

but does not constitute evidence against a true increase in the incidence of the disease during the whole, or a more recent part, of the last 100 years.

Hueper (*43*), accepting a true increase in the incidence of lung cancer, regards an increase dating back to 1900, or before the widespread use of cigarettes, as evidence against the cigarette–lung-cancer relationship. His contention would have crucial import only if it were maintained that cigarette smoking is the sole cause of lung cancer.

The vital statistics and the necropsy data that support the presumption of a real increase in lung-cancer risk certainly apply to the years after 1920. Because of the uncertainties associated with changes in diagnostic accuracy, no firm conclusions can be reached on whether the *rate* of increase in lung-cancer mortality has, in truth, accelerated since 1920.

Effect of Aging

Rosenblatt (*67*) has raised the question about the effect of the aging population on the lung-cancer rate. This particular point has been investigated by the use of age-adjusted rates. Dunn (*22*) has noted that only one sixth of the over-all increase in lung-cancer mortality among males in the United States (from 4 to 24 deaths per 100,000 males between 1930 and 1951) could be attributed to an aging population. Similar findings (*16*) have been presented for England and Wales where observations on lung-cancer mortality date back to 1900; the 1953 mortality rate for both sexes, 34 per 100,000 population, was 43 times the corresponding 1900 rate, 0.8 per 100,000 population. Allowance for increased average age of the population could account for only half this rise in lung-cancer mortality, with a 24-fold difference between 1900 and 1953.

Also, an aging population does not affect the age-specific death rates and cannot account for the phenomenon of increasingly higher lung-cancer mortality at all ages throughout the lifespan, which has occurred among successively younger groups of males born in the United States and England and Wales since 1850. A similar but less pronounced "cohort displacement" has been shown for females.

Diagnostic Factors

Little (*52*) and others (*64*) have raised the important question on whether better diagnostic measures and more complete reporting have resulted in a spurious increase in the recorded attack rate. Several special features of the increase in lung-cancer mortality would be difficult to account for on diagnostic grounds. These include the continuous rising ratio of male to female deaths, the increasing lung-cancer mortality rate among successively younger cohorts, and the magnitude of the current, continuing, increase in lung-cancer mortality (*16*). By 1955, among white males, 50 to 64 years of age, in the United States, more deaths were attributed to lung cancer than to all other respiratory diseases combined.

Gilliam (*29*) has made a careful study of the potential effect of improved diagnosis on the course of the lung-cancer death rate. Even assuming

that 2 percent of the deaths certified in past years as tuberculosis or other respiratory disease were really due to lung cancer, he concluded that " . . . all of the increase in mortality attributed to cancer of the lung since 1914 in United States white males and females cannot be accounted for by erroneous death certification to other respiratory diseases without unreasonable assumptions of age and sex differences in diagnostic error." His computations reduced the respective 26-fold and sevenfold increase in lung-cancer mortality among males and females, between 1914 and 1950, to the more modestly estimated dimensions of fourfold and 30 percent, respectively. These estimates are certainly the lower bound on the magnitude of the true rate of increase during this period.

The Copenhagen Tuberculosis Station data, examined by Clemmesen *et al.* (*14*), provide the greatest measure of control on the diagnostic improvement factor. In a tuberculosis referral service, used extensively by local physicians, where diagnostic standards and procedures including systematic bronchoscopy remained virtually unchanged between 1941 and 1950, the lung-cancer prevalence rate among male examinees increased at a rate comparable to that recorded by the Danish cancer registry for the total male population. This can be regarded as evidence that the reported increase in Danish incidence is not due to diagnostic changes.

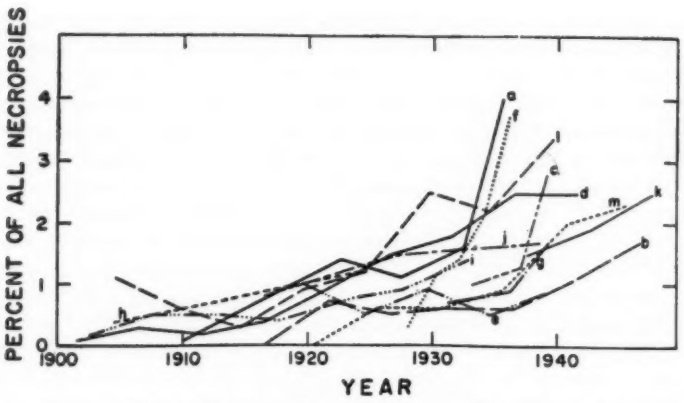
Necropsy Data

Most necropsy data agree with mortality data on the increase in lung-cancer risk. To establish this point we referred to a necropsy series summarized by Steiner (*72*), and returned to the original sources for evidence on the nature of changes over time. Since an existing compilation was chosen, the results do not represent a culling of autopsy series for data favorable to this thesis. The findings from 13 series are summarized in text-figure 1 as the proportion of lung cancers in relation to all necropsies. The relative frequency in terms of total tumors or total carcinomas yielded results which would lead to substantially the same inferences.

Mortality and necropsy data have their own virtues and weaknesses. Death certificates provide a complete report of deaths, but do not emphasize a high quality of diagnostic evidence, while the reverse holds true for necropsies. However, since both approaches lead to the same inferences, neither great variation in the quality of diagnostic evidence nor the unrepresentative nature of some of the necropsy observations can be viewed as plausible interpretations of the results. The alternative conclusion of a real increase in lung-cancer risk remains.

Urban-Rural Differences

Emphasis has been placed on the alleged incompatibility of the excess lung-cancer mortality, among urban residents, with the cigarette-smoking hypothesis (*43*, *44*, *54*). Mortality data from several countries indicate strongly that lung-cancer rates are much higher in cities than in rural areas, and the observation that urban males in general have higher lung-



TEXT-FIGURE 1.—Relative frequency of carcinomas of the lung found at necropsy in 13 series. Complete references to material shown are listed in Steiner (*72*): (a) Frissell, (b) Gibson, (c) Halpert, (d) Jeuther, (e) Johnson, (f) Matz, (g) Menne, (h) Nagayo, (i) Perrone, (k) Saxton, (l) Steiner (1944), (m) Steiner (1950).

cancer mortality than rural males is undoubtedly correct. The assertion of Macdonald (*54*) that ". . . country people smoke as much, if not more, than do city people . . ." is not borne out by the facts (*35*). Nevertheless, the evidence indicates that adjustment for smoking history could account for only a fraction of this urban-rural difference (*33*).

However, this does not establish the converse proposition that control of residence history in the analysis of collected data would account for the excess lung-cancer risk among cigarette smokers. Evidence now in hand weighs strongly against this last assertion. Stocks and Campbell (*74*), in their report on lung-cancer mortality among persons in Liverpool, the suburban environs, and rural towns in North Wales, showed that heavy smokers have higher lung-cancer rates when urban and rural males were studied separately. Mills and Porter (*58*) reported similar findings in Ohio. These results agree with the experience of the Hammond-Horn (*38*) study, which revealed markedly higher death rates for bronchogenic carcinoma among smokers regardless of whether they lived in cities or in rural areas. No contradictory observations are known to us.

Sex Differences

The sex disparity in lung-cancer mortality has also been cited (*25*, *54*) as grounds for discarding the cigarette-smoking hypothesis. In this connection it should be noted that persons advocating this line of argument have minimized sex differences in smoking habits to a degree not supported by available facts. A survey of smoking habits in a cross section of the United States population (*35*) demonstrated that men, on the average, have been smoking for longer periods than women. The sex differences in tobacco use were especially pronounced at ages over 55, when most lung-cancer deaths occur; 0.6 percent of United States females in this age

group have been reported as current users of more than 1 pack of cigarettes daily compared to 6.9 percent of United States males. British data (*76*) also revealed much lower tobacco consumption among females, particularly for the years before World War II.

The present data contrasting the experience by sex would appear to support the cigarette-smoking hypothesis rather than discredit it. When differences in smoking habits are considered, it is possible to reduce the observed fivefold excess lung-cancer mortality among males to the 40 percent excess mortality which prevails for many other causes of death (*33*). One intriguing finding from these studies is that the estimated death rates for female nonsmokers agree closely with the death rates derived from retrospective studies on male nonsmokers (*34*).

Evidence for Other Etiologic Factors

Etiologic factors of industrial origin, such as exposure to chromates and coal gas, are well established (*16*). Excess lung-cancer risks among such groups as asbestos workers who develop asbestosis, appear likely (*16*). One epidemiologic study (*11*) of British, World War I, veterans exposed to mustard gas and/or with a wartime history of influenza revealed virtually no excess lung-cancer risk among these groups.

The existence of other important lung-cancer effects associated with such characteristics as socioeconomic class cannot be questioned. Cohart (*15*) found that the poorest economic class had a 40 percent higher lung-cancer incidence than the remaining population of New Haven, Connecticut. Results from the 10-city morbidity survey (*21*) have revealed a sharp gradient in lung-cancer incidence, by income class, for white males, which is consistent with Cohart's findings. Since cigarette smoking is not inversely related to socioeconomic status, we can agree with Cohart ". . . that important environmental factors other than cigarette smoking exist that contribute to the causation of lung cancer." These and other findings are convincing evidence for multiple causes of lung cancer. It is obviously untenable to regard smoking of tobacco as the sole cause of lung cancer.

Two points should be made: The population exposed to established industrial carcinogens is small, and these agents cannot account for the increasing lung-cancer risk in the remainder of the population. Also, the effects associated with socioeconomic class and related characteristics are smaller than those noted for smoking history, and the smoking-class differences cannot be accounted for in terms of these other effects.

Special Population Groups

Haag and Hanmer (*32*) reported that employees in 9 processing plants of the American Tobacco Company, with an above-average proportion of smokers, had a lower mortality than the general population of Virginia and North Carolina for all causes and for cancer and cardiovascular diseases, but no higher mortality for respiratory cancer and coronary disease. They concluded: "The existence of such a population makes

it evident that cigarette smoking *per se* is not necessarily or invariably associated with a higher risk of lung cancer or cardiovascular diseases or with diminished longevity."

The group studied by Haag and Hanmer was too small to yield significant results on respiratory cancer. Moreover, a major flaw in the conclusion has been pointed out by Case (*10*). It is well known that mortality comparisons cannot be drawn directly between employee groups and the general population, since the death rates for many groups of employed persons are lower than death rates for the general population with age, sex, and race taken into consideration. This is true because there is a strong tendency to exclude from employment those persons who have acute or chronic diseases or who are seriously disabled from any cause and those employees who develop permanent disabilities from disease or other causes are usually discharged, retired, or dropped from the list of regular employees. Reasons of this nature undoubtedly account for the deficit in deaths from all causes noted in the group of employees under consideration.

A different picture is provided by the Society of Actuaries (*71*) who made a study for 1946 through 1954. The death claims for employees of the tobacco industry were reported to be slightly higher than, and the permanent disability claims were reported to be over three times as high as, those for employees in nonrated industries as a whole. This latter comparison indicates that the basic assumption of the Haag and Hanmer study is incorrect. Also, interpretation of group comparisons in this field should account separately for the experience of smokers and nonsmokers. We hope that Haag and Hanmer will supplement the report to provide data for smokers and nonsmokers in the study population.

II. Retrospective and Prospective Studies

The association between smoking and lung cancer has now been investigated and reported by at least 21 independent groups of investigators in 8 different countries, who employed what is known as the retrospective method (*16*, *54*, *68*, *69*, *73*, *75*). In these studies, patients with lung cancer, or their relatives, were questioned about their smoking history and other past events, and the answers compared with those of individuals without lung cancer who were selected as controls. Although these 21 studies have certain features in common, they varied greatly in the methods of selecting the groups, the methods of interview, and other important aspects.

The association between smoking and lung cancer was further investigated in two countries by three independent groups (*18*, *20*, *38*), using the prospective method. In these studies, large groups were questioned on smoking habits and other characteristics, and the groups were observed for several years for data on mortality and causes of death. The three prospective studies also varied in several important details including the type of subjects, the selection of subjects, and the method of obtaining information on smoking habits.

In each of these studies, an association was found between smoking and lung cancer. In every investigation where the type of smoking was considered, a higher degree of association was found between lung cancer and cigarette smoking than between lung cancer and pipe or cigar smoking. In every instance where amount of smoking was considered, it was found that the degree of association with lung cancer increased as the amount of smoking increased. When ex-cigarette smokers were compared with current cigarette smokers, it was found that lung-cancer death rates were higher among current cigarette smokers than among ex-cigarette smokers.

A number of investigators (*3*, *36*, *54*) have criticized the retrospective method but, for the most part, the specific points of criticism apply only to some of the studies and not to others. Some features of the three prospective studies on smoking also have been criticized. Again, certain of the points of criticism apply to one or another of the three prospective studies but not to all three. Specifically, doubts raised as to the validity of the early findings of the prospective studies have been eliminated by the persistence of the findings in the later phases of the same studies.

The validity of the findings on these extensive investigations has been questioned in regard to two major aspects: 1) the methods of selection of the study groups, and 2) the accuracy of information regarding smoking habits and the diagnosis of lung cancer.

Selection of Study Groups

Neyman (*60*) pointed out that a study based on a survey of a population at some given instant of time may yield misleading results. Suppose that a study is made on a day when all patients with lung cancer and a group of people without lung cancer are questioned about their smoking habits. If smokers with lung cancer live longer than nonsmokers with lung cancer, there would be a higher proportion of smokers in the lung-cancer group than in the control group—this would follow without questioning the proposition on which the model is based. However, only two of the retrospective studies were conducted in a way approximating an "instantaneous survey" procedure, so that this criticism does not apply to most of the studies. Furthermore, this difficulty is completely avoided in prospective studies.

Berkson (*3*) indicated that people with two specific complaints are more likely to be hospitalized than people with only one of these complaints. If a retrospective study were conducted exclusively on hospital patients an association would be found between these two specific complaints, even if there were no association between the same two complaints in the general population. This would influence the results if smokers with lung cancer are more likely to be hospitalized than nonsmokers with lung cancer. However, Berkson showed that this difficulty is trivial if a high percentage of people with either one of these two conditions is hospitalized, which is the situation with lung-cancer patients. Furthermore, one retrospective study (*74*) included all lung-cancer patients who were in the study area, including those not hospitalized; another retrospective study (*82*) was

based on individuals who died of lung cancer and other diseases regardless of whether thay had been hospitalized or not. This difficulty does not arise in prospective studies.

In all but one of the 21 retrospective studies, the procedure was to compare the smoking habits of lung-cancer patients with the smoking habits of a control group who did not have lung cancer. Hammond (*36*), Berkson (*3*), and others have pointed out the grave danger of bias if the control group is not selected in such a way as to represent (in respect to smoking habits) the general population which includes the lung-cancer patients. Subsequent events have proved that this criticism is well founded, though the direction of the bias in most studies turned out to yield an underestimate of the degree of association between cigarette smoking and lung cancer. The reason was that in most of the retrospective studies the control group consisted of patients with diseases other than lung cancer. The choice of such a control group is tantamount to assuming that there is no association between smoking and diseases which resulted in hospitalization of the control subjects. This was an incorrect assumption since other studies have indicated an association between smoking and a number of diseases, such as coronary artery disease, thromboangiitis obliterans, and cancer of the buccal cavity.

Doll and Hill (*17*), recognizing the possibility of bias in a control group selected from hospital patients, obtained an additional control group by ascertaining the smoking habits of the general population in a random sample of the area in which their hospital was located. The largest percentage of smokers (particularly heavy smokers) was found in the lung-cancer group, the smallest percentage of smokers was found in the general population sample, and an intermediate percentage of smokers was found in the hospital-control group. Similar results have been reported in a recent study of women (*34*).

Berkson (*3*) pointed out that the criticisms in regard to selection bias in the retrospective studies are also applicable to the earlier findings in a prospective study. Suppose that, in selecting subjects for a prospective study, sick smokers are overrepresented in relation to well smokers and/or well nonsmokers are overrepresented in relation to sick nonsmokers. In this event, during the earlier period after selection, the death rate of the smokers in the study would be higher than the death rate of the nonsmokers in the study, even if death rates were unrelated to smoking habits of the general population. If smoking is unrelated to death from lung cancer (or other causes), the death rate of the smokers would tend to equalize with that of the nonsmokers as the study progressed. Thus, the bias would diminish with time, and a relationship due to such bias would disappear. This general principle is well known to actuaries and is one of the cornerstones of the life insurance business.

Hammond and Horn (*38*), recognizing this possible difficulty, excluded from the study all persons who were obviously ill at the time of selection. As expected, the total death rate of the study population was low and very few deaths from lung cancer occurred during the first 8 months after

selection. The total death rate, and particularly the death rate from lung cancer, rose considerably in the subsequent 3 years. What is more important, the observed association between cigarette smoking and lung cancer was considerably higher in the latter part than in the early part of the study, and the association between cigarette smoking and total death rates was also somewhat greater in the latter part of the study. This showed that the original bias in the selection of the subjects was slight and that it yielded an underestimate of the degree of association between smoking and death rates.

This particular problem was not encountered in the prospective studies of Doll and Hill (*18*) who could observe the death rates of all physicians in Great Britain (nonresponders as well as responders to the smoking questionnaire). The prospective study of Dorn (*20*) also had a defined population of veterans holding insurance policies, and nonresponders were observed as well as responders. Moreover, these two studies also showed that higher mortality from lung cancer among smokers was more evident during the later period than in the earlier period of observation. Thus, in the course of time, there was no disappearance of any selection bias factors that may have been introduced into the original study groups.

The subjects for the Hammond and Horn prospective study (*38*) were selected by volunteer workers with specific instructions on how it should be done. Mainland and Herrera (*55*) have suggested that the volunteer workers may have introduced a bias in the way they selected the subjects. The foregoing evidence of persistence and accentuation of the differences between smokers and nonsmokers, in time, effectively counters purposeful, as well as unknown, sources of such selection.

Accuracy of Information

Berkson (*3*, *4*) has remarked that the two major variables considered in all these studies—the ascertainment of smoking habits and the diagnosis of disease—are both subject to considerable error. The accuracy of diagnosis is not a major problem in retrospective studies because the investigator can restrict his study to those patients whose diagnosis of lung cancer has been thoroughly confirmed. This feature has been taken into consideration in several retrospective studies. It is more of a problem in prospective studies since all deaths that occur must be included, and certainly some of the diagnoses will be uncertain. However, in all three prospective studies, the *total death rate* was found to be higher in cigarette smokers than in nonsmokers and found to increase with the amount of cigarette smoking. If some of the excess deaths associated with cigarette smoking and ascribed to lung cancer were actually due to some other disease, then it means that: *a*) the association between cigarette smoking and lung cancer was somewhat overestimated, but *b*) the association between smoking and some other disease was somewhat underestimated. The reverse would be true if some of the excess deaths associated with cigarette smoking and ascribed to diseases other than lung cancer were actually due to lung cancer. Hammond and Horn (*38*)

found that the association with cigarette smoking was greater for patients with a well-established diagnosis of lung cancer than for patients with less convincing evidence for a diagnosis of lung cancer. This suggests that inaccuracies in diagnosis resulted somewhat in an underestimate of the degree of association between smoking and lung cancer.

The study on physicians, by Doll and Hill (*18*), in which presumably the clinical and pathologic evidence of the cause of death would be somewhat more than in the general population considered by Hammond and Horn and by Dorn, yields almost identical risks to lung cancer by smoking class.

In regard to information about smoking, Finkner *et al.* (*24*) have made a thorough study of the accuracy of replies to questionnaires on smoking habits. Their results indicate that replies are not completely accurate but that most of the errors are relatively minor—very few heavy smokers are classified as light smokers. Random and independent errors simply tend to diminish the apparent degree of association between two variables. A national survey of smoking habits in the United States (*35*) yielded results on tobacco consumption that were consistent with figures on tobacco production and taxation.

On two occasions several years apart, Hammond and Horn (*38*) and Dorn (*20*) questioned a proportion of their subjects. The results indicated close reproducibility in the answers.

Hammond (*36*) and others (*54*) have questioned the reliability of the retrospective method on the grounds that the illness may bias the responses given by the patient or his family when they are questioned about smoking habits, and that knowledge of the diagnosis may bias the interviewer. This possible difficulty was minimized in several of the 21 retrospective studies on smoking in relation to lung cancer. For example, in the study conducted by Levin (*49*), all patients admitted to a hospital during the course of several years were questioned about their smoking habits *before* a diagnosis was made. Only a small proportion later turned out to have lung cancer, though many had lung disease symptoms or lung diseases other than lung cancer. Doll and Hill (*18*) also showed that patients whose diagnosis of lung cancer was subsequently established to be erroneous had smoking histories characteristic of the control rather than of the lung-cancer group. Furthermore, a larger percentage of cigarette smokers have been found among patients with epidermoid carcinoma of the lungs than among patients with adenocarcinoma of the lungs (*34*, *46*, *79*). This could hardly have resulted from bias either on the part of the patient or on the part of the interviewer.

Multiple Variables

Arkin (*1*), Little (*52*), Macdonald (*54*), and others have criticized the studies of cigarette–lung-cancer relationship on the grounds that only smoking habits were really investigated, and that numerous other possible variables were not considered.

This criticism may seem especially appropriate in view of the accepted fact that no single etiologic factor has been proposed for any neoplastic

disease. The criticism may also be valid in relation to any one of the retrospective and prospective studies. However, in the aggregate, quite a number of other variables have been specifically investigated or can be inferentially derived. Of course, all studies considered the basic factors of age and sex; some dealt with geographic distribution (*74*), occupation (*8*), urban or rural residence (*74*), marital and parous status (*34*), and some other habits such as coffee consumption (*34*).

The Doll and Hill (*18*) prospective study was confined to a single professional group, physicians. Thus there could be no great variation attributable to occupation or socioeconomic status. Stocks and Campbell (*74*) put particular emphasis on the study of air pollution and occupational exposure and included a number of other factors in addition to smoking. It is evident, in the Hammond-Horn (*38*) study and other investigations, that there is a consistent relationship between urban residence and a higher mortality due to lung cancer. The important fact is that in all studies, when other variables are held constant, cigarette smoking retains its high association with lung cancer.

The only factors that may show a higher correlation with lung cancer than heavy cigarette smoking are such occupations as those of the Schneeberg miners and manufacturers of chromate (*16*). We are not acquainted with actual studies of these and related occupation groups in which cigarette and other tobacco consumption is also considered. Such studies, we suggest, would be useful additions to our knowledge of other etiologic agents and of the interplay between multiple causes in human pulmonary cancer.

III. Studies on Pathogenesis

Inhalation of Smoke

If cigarette smoking produces cancer of the lungs as a result of direct contact between tobacco smoke and the bronchial mucosa, smokers who inhale cigarette smoke should be exposed to higher concentrations of the carcinogens than noninhalers and therefore have a higher risk to the development of lung cancer. The retrospective study of Doll and Hill (*17*), however, elicited no difference between patients with lung cancer and the controls in the proportion of smokers who stated that they inhaled. Fisher (*25*), Hueper (*43*), and Macdonald (*54*) have emphasized this point as contradictory to the smoking–lung-cancer relationship, and, of course, it is. Unfortunately, this particular finding was not reinvestigated in the prospective study of Doll and Hill (*18*).

Three authors, Lickint (*50*), Breslow *et al.* (*8*), and Schwartz and Denoix (*68*), however, *did* find the relative risk of lung cancer to be greater among inhalers than among noninhalers when age, type, and amount of smoking were held constant. It must be admitted that there is no clear explanation of the contradiction posed by the Doll-Hill (*17*) findings, though a number of plausible hypotheses could be advanced. More experimental work is required, including some objective definition and measurement of the depth and length of inhalation.

Hammond (*37*) has recently queried male smokers about their inhalation

practices. He found that very few pipe and cigar smokers inhale; that most men inhale who smoke only cigarettes; and that there are proportionally fewer inhalers among men who smoke both cigars and cigarettes than among men who smoke only cigarettes. These findings are compatible with the view that differences in inhaling account for the fact that the lung-cancer death rate of cigar and pipe smokers is less than the lung-cancer death rate of cigarette smokers; and that the lung-cancer death rate of men who smoke both cigars and cigarettes is somewhat lower than the lung-cancer death rate of men who smoke only cigarettes.

Upper-Respiratory Cancer

Rosenblatt (*67*) has drawn attention to the fact that increased consumption of cigarettes has not been accompanied by an increase in upper-respiratory cancer similar to that noted in cancer of the lung and bronchus. Hueper (*43*) also has expressed doubts about the causative role of cigarette smoking on the basis that cigarette smoking is not associated with cancer of the oral cavity or of the fingers, which are often stained with tobacco tar.

The premise that a carcinogen should act equally on different tissues is not supported by experimental or clinical evidence (*70*). Carcinogens, which produce liver tumors in animals, may be noncarcinogenic when applied to the skin. Coal soot, accepted as etiologically related to carcinoma of the scrotum in chimney sweeps, does not increase the risk to cancer of the penis. There is no *a priori* reason why a carcinogen that produces bronchogenic cancer in man should also produce neoplastic changes in the nasopharynx or in other sites. It is an intriguing fact, deserving further research, that carcinoma of the trachea is a rarity, whereas carcinoma of the bronchus is common among individuals exposed to chromates, as well as among chronic cigarette smokers.

Several studies have established the association of all types of tobacco smoking, including cigarettes, with cancer of the oral cavity (*81*). However, the *relative* risk of developing cancer of the mouth is greater for cigar and pipe smokers than for cigarette smokers. The risk of laryngeal cancer is increased by smoking and an equal risk exists among cigarette, cigar, and pipe smokers (*80*). The per capita consumption of cigars and pipe tobacco has decreased since 1920, while cigarette smoking has increased (*59*).

These associations contrast sharply with the findings on lung cancer, which have consistently shown that cigarette smokers have much higher risks than either cigar or pipe smokers. Since 1920 the increase in tobacco consumption has been primarily due to the rise in cigarette consumption (*59*), and the stabler rates for intra-oral and laryngeal cancer, while the lung-cancer rates have increased steeply, can be considered compatible with the causal role of cigarette smoking in lung cancer.

Effect of Tobacco Smoke on Bronchial Mucosa

Statements by Hartnett (*39*), Macdonald (*54*), and others (*3*, *52*) imply that the relationship of cigarette smoking and lung cancer is based ex-

clusively on "statistics" and lacks "experimental" evidence. The differentiation between various methods of scientific inquiry escapes us as being a valid basis for the acceptance or the rejection of facts. Nevertheless it is true that historically the retrospective studies on lung cancer preceded the intensive interest in laboratory investigations stimulated by the statistical findings.

Hilding (*40*) has shown experimentally that exposure to cigarette smoke inhibited ciliary action in the isolated bronchial epithelium of cows. Kotin and Falk (*45*) obtained essentially the same results in experiments on rats and rabbits. Hilding (*41*) further showed that inhibition of ciliary action interfered with the mechanism whereby foreign material is ordinarily removed from the surface of bronchial epithelium. In addition, he found that foreign material deposited on the surface tended to accumulate in any area where the cilia have been destroyed. Auerbach *et al.* (*2*) found that the small areas of the bronchial epithelium where ciliated columnar cells were absent appeared more frequently in smokers than in nonsmokers. Chang (*12*) found that cilia were shorter, on an average, in the bronchial epithelium of smokers than in that of nonsmokers.

These studies have demonstrated the existence of a mechanism whereby foreign material from any source (*e.g.*, tobacco smoke, industrial dusts, fumes from automobile exhausts, general air pollutants, and, perhaps, pathogenic organisms) is likely to remain in contact with the bronchial epithelium for a longer period in smokers than in nonsmokers.

Auerbach and his associates (*2*) studied the microscopic appearance of the bronchial epithelium of patients who died of lung cancer and patients who died of other diseases. Each of these two groups of patients was classified according to whether they were nonsmokers, light smokers, or heavy cigarette smokers. Among the cancer patients there were no nonsmokers. Approximately 208 sections from all parts of the tracheobronchial tree from each patient were examined. Many areas of basal-cell hyperplasia, squamous metaplasia, and marked atypism with loss of columnar epithelium were found in the tracheo-bronchial tree of men who had died of lung cancer. Almost as many such lesions were found in heavy cigarette smokers who had died of other diseases; somewhat less were found in light cigarette smokers; and much less in nonsmokers. Chang (*12*) has reported similar findings in the bronchial epithelium of smokers compared with nonsmokers.

The chief criticism of Auerbach's study has concerned terminology. Following the definition previously set forth by Black and Ackerman (*5*), Auerbach *et al.* used the term "carcinoma-*in-situ*" to describe certain lesions with marked atypical changes and loss of columnar epithelium. Whether this is an appropriate term may be questioned, but it is not relevant to the validity of the findings. Certainly there are no data to indicate what proportion of these morphologically abnormal areas would progress to invasive carcinoma.

The recent findings of Auerbach *et al.* and Chang have been reproduced experimentally in animals. Rockey and his associates (*66*) applied tobacco

"tar" directly to the bronchial mucosa of dogs. Within 3 to 6 weeks, the tar-treated surface became granular and later developed wartlike elevations. Upon microscopic examination, hyperplasia, transitional metaplasia, and squamous metaplasia were found in these areas. Leuchtenberger *et al.* (*48*) exposed mice to cigarette smoke for periods up to 200 days. The bronchial epithelium was then examined microscopically. Bronchitis, basal-cell hyperplasia, and atypical basal-cell hyperplasia were found in the majority of the animals, and squamous metaplasia in a few. Further work and longer periods of observation are necessary to establish whether some of these lesions would progress to frank neoplasia.

IV. Other Laboratory Investigations

Skin Cancer in Rodents

One of the links in the total evidence for the causal relationship of cigarette smoking and lung cancer is the demonstration that tobacco-smoke condensates (usually referred to as "tars") have the biologic property of evoking carcinoma in certain laboratory animals, particularly mice. The production of skin cancer in mice, following repeated, long-term applications of tobacco tar, has now been reported from at least six different laboratories (*7*, *23*, *28*, *61*, *63*, *83*). It is undeniable that some investigators did not obtain positive results, perhaps because the dose and other experimental conditions were different, or because the complex tobacco tars probably varied widely in their composition. The negative results of Passey *et al.* (*62*) have been quoted by Hueper (*43*) and others, but a more recent experiment by Passey (*63*) with Swiss strain mice did lead to the appearance of at least two carcinomas after repeated applications of tobacco-smoke condensate.

Little (*52*) indicated that ". . . the extrapolation to the human lung of results obtained by painting of or injection into the skin of mice is decidedly questionable." Direct extrapolation from one species to another is, of course, not justified. Nevertheless, results in animals are fully consistent with the epidemiologic findings in man. A quotation from Kotin (*44*) is appropriate: "The chemical demonstration of carcinogenic agents in the environment and their successful use for the production of tumors in experimental animals do not prove or even especially strongly suggest a like relationship in the instance of man. When, however, a demonstrable parallelism exists between epidemiologic data and laboratory findings, greater significance accrues to both. Medical history is replete with examples in which laboratory findings have been proved ultimately to have their counterpart in the human experience. Exceptions have been very few."

Greene (*31*), while discounting the significance of the induction of skin carcinoma in Swiss mice because of the constitutionally "high differential susceptibility" of the strain, believes that the failure to induce neoplasms in embryonic transplants exposed to tobacco tar is more important evidence. Greene's interesting technique does produce positive results

when pure chemicals such as benzo[*a*]pyrene are used, and this chemical has been recovered from some samples of tobacco-smoke condensate. We are not acquainted with reports of neoplasms arising in embryonic tissue that has been exposed *in vitro* to coal tar, another crude mixture that contains carcinogens.

The high frequency of carcinoma induction reported by Wynder *et al.* (*83*) has not been achieved by other investigators, who reported that no more than 20 percent of animals, and usually considerably less, developed carcinoma of the skin. The presence of cocarcinogenic materials in tobacco-smoke condensates has been demonstrated by Gellhorn (*28*) and by Bock and Moore (*7*). To the mouse data are now added the data on the induction of skin cancer in some rabbits painted with tobacco-smoke condensate (*30*); this condensate, when combined with a killed suspension of tubercle bacilli, and introduced into a bronchus, produced a carcinoma of the bronchus in one rat (*6*).

Since malignant neoplasms have been obtained in several strains of mice, and a few neoplasms have been produced in rabbits and rats, the issue of strain or species limitation to the reaction is more difficult to maintain. It is, of course, a fact that many agents shown to be carcinogenic to the skin of mice have not been proved carcinogenic to man. In most instances there is simply no experience with such agents in man, so that lack of proof really represents lack of data, pro and con.

The Problem of Dosage

Little (*52*) has further questioned the applicability of animal data to man, as follows: "Tobacco smoke or smoke condensate has failed to produce cancer even on the skin of susceptible strains of mice when applied in the quantity and at an exposure rate that would simulate conditions of human smoking."

The differences in species, tissues, and conditions between the induction of neoplasms on the skin of mice and in the bronchi of man preclude fine comparisons of dose and time relationships.

Bronchogenic Cancer in Animals

The pulmonary adenomatous tumor in mice, rats, and guinea pigs cannot be compared with the bronchogenic carcinoma in man (*70*). Until a few years ago, the experimental induction of epidermoid carcinoma had been achieved only in a few mice by passing strings impregnated with carcinogenic hydrocarbons through the lung. Epidermoid carcinoma of the lung was consistently produced in rats by beryllium (*78*), by carcinogenic hydrocarbons introduced as fixed pellets into bronchi of rats (*47*), and by inhalation of radioactive particles (*13*).

Little (*52*) has noted that ". . . prolonged exposure of the lungs of rodents to massive doses of cigarette smoke has failed to produce bronchogenic cancer." This remains true at the time of this report, although it can be questioned whether any animal receives as large a dose of cigarette smoke through indirect exposure as a human being does by voluntary

deep inhalation. Therefore the failure may be a technical one, which may be solved by further experimentation. The early results of Leuchtenberger *et al.* (*48*) suggested that this may be achieved.

Carcinogens in Tobacco Smoke

The isolation and identification of specific chemical constituents in tobacco smoke, which are carcinogenic for the pulmonary tissue of man, is an important area for research.

It has been clear for some time that combustion or pyrolysis of most organic material, including tobacco, will form higher aromatic polycyclics of established carcinogenic activity (*85*). A number of higher aromatic polycyclics have been identified and isolated (*53*, *61*, *77*, *84*). These materials include benzo[*e*]pyrene, benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene, chrysene, and, most recently, a newly established carcinogen, 3,4-benzfluoranthene. Whether these compounds are equally involved in human pulmonary carcinogenesis is, of course, conjectural.

Little (*52*) has implied that a specific constituent must be found to account for the biologic activity of tobacco smoke. This is not necessary. The situation is similar to the establishment of the carcinogenic activity of tar, which was accepted before the isolation of benzo[*a*]pyrene by Kennaway and his coworkers. In this instance, also, benzo[*a*]pyrene is most probably not the only carcinogen in the complex mixture called tar, and there are strong indications that some noncarcinogenic components in tar may have cocarcinogenic effects.

V. Interpretation

Three interpretations of the observed association of lung cancer and cigarette smoking are possible: 1) that cigarette smoking "causes" lung cancer, either (*a*) through the direct carcinogenic action of smoke on human bronchial epithelium or (*b*) by a more indirect mode of action such as making the individual susceptible to some other specific carcinogenic agent in the environment; 2) that lung cancer "causes" cigarette smoking, perhaps because a precancerous condition sets up a process which leads to a craving for tobacco; 3) that cigarette smoking and lung cancer both have a common cause, usually specified as a special constitutional make-up, perhaps genetic in origin, which predisposes certain individuals to lung cancer and also makes them cigarette smokers.

The second hypothesis was advanced by Fisher (*26*), apparently for the sake of logical completeness, and it is not clear whether it is intended to be regarded as a serious possibility. Since we know of no evidence to support the view that the bronchogenic carcinoma diagnosed after age 50 began before age 18, the median age at which cigarette smokers begin smoking, we shall not discuss it further.

The Constitutional Hypothesis

The first hypothesis may be referred to as the *causal* hypothesis and the third as the *constitutional* hypothesis. Nothing short of a series of

independently conducted, controlled, experiments on human subjects, continued for 30 to 60 years, could provide a clear-cut and unequivocal choice between them. We nevertheless argue that evidence, in addition to that associating an increased mortality from lung cancer with cigarette smoking, is entirely consistent with the causal hypothesis but inconsistent, in many respects, with the constitutional hypothesis, so that even in the absence of controlled experimentation on human beings the weight of the evidence is for the one and against the other.

The difficulties with the constitutional hypothesis include the following considerations: (*a*) changes in lung-cancer mortality over the last half century; (*b*) the carcinogenicity of tobacco tars for experimental animals; (*c*) the existence of a large effect from pipe and cigar tobacco on cancer of the buccal cavity and larynx but not on cancer of the lung; (*d*) the reduced lung-cancer mortality among discontinued cigarette smokers. No one of these considerations is perhaps sufficient by itself to counter the constitutional hypothesis *ad hoc* modification of which can accommodate each additional piece of evidence. A point is reached, however, when a continuously modified hypothesis becomes difficult to entertain seriously.

Changes in Mortality

Mortality from lung cancer has increased continuously in the last 50 years, and considerably more for males than females. Such an increase can be explained either as the result of an environmental change (to which males are more exposed or more sensitive than females, if both are equally exposed) or as the result of a sex-linked mutation. The constitutional hypothesis must be modified in the light of this increase, since an unchanging constitutional make-up cannot by itself explain an increase in mortality. Proponents of the constitutional hypothesis have not indicated the type of modification they would consider. Three suggest themselves to us: 1) differences in constitutional make-up are genetic in origin, but rather than predisposing one to lung cancer, they make one sensitive to some new environmental agent (other than tobacco), which does induce lung cancer; 2) differences in constitutional make-up are not genetic but are the result of differential exposure to some new environmental agent, which both predisposes to lung cancer and creates a craving for cigarette smoke; 3) the mutation has led to a greater susceptibility to lung cancer and a preference for cigarette smoke.

In the first two situations the effect of the postulated constitutional make-up would be mediated through an environmental agent. The modified hypothesis thus requires the existence of an environmental agent other than tobacco, exposure to which would be at least as highly correlated with lung-cancer mortality as exposure to cigarettes, and which also would be highly correlated with cigarette consumption. No such agent has yet been found or even suggested. In view of the magnitude of the increase in mortality from lung cancer, the third situation would require a mutation rate exceeding anything previously observed.

Experimental Carcinogenesis With Tobacco Tar

Condensed tobacco smoke contains substances that are carcinogenic for mouse and rabbit skin. It does not necessarily follow that these substances are also carcinogenic for human lungs nor does it follow that they are not. However, the constitutional hypothesis asserts they are not; and that it is simply a coincidence that these materials which are carcinogenic for experimental animals are also associated with a higher lung-cancer mortality in man.

Types of Tobacco and Cancer Site

A greatly increased lung-cancer risk is associated with increased cigarette consumption but not with increased consumption of pipe and cigar tobacco. Studies on cancer of the buccal cavity and larynx, however, have demonstrated a considerably higher risk among smokers, irrespective of the form of tobacco used. Only two ways of modifying the constitutional hypothesis to take account of this evidence occur to us: 1) There are two different constitutional make-ups, one of which predisposes to cigarettes but not to pipe and cigar consumption and to cancer of the lung, and the other predisposes to cancer of the buccal cavity and larynx but not of the lung and to tobacco consumption in any form. 2) Constitutional make-up predisposes to cigarette consumption and lung cancer only, but tobacco smoke, whether from cigarettes, cigars, or pipes, is carcinogenic for the mucosa of the buccal cavity and the larynx but not for the bronchial epithelium.

Mortality Among Discontinued Smokers

Mortality from lung cancer among discontinued cigarette smokers is less than that among those continuing to smoke (*18*, *38*); the magnitude of the reduction depending on amount previously smoked and the length of the discontinuance. The hypothetical constitutional factor which predisposes to lung cancer and cigarette smoking cannot therefore be a constant characteristic of an individual over his lifetime but must decrease in force at some time in life, thus resulting in the cessation of cigarette smoking and a concomitant, but not causally related, reduction in the lung-cancer risk. Furthermore, since cigarette smoking is rarely begun after age 35 (*35*), it must be inferred that the constitutional factor cannot increase in force with the passage of time, even though it may decrease.

In summary, the constitutional hypothesis does not provide a satisfactory explanation of all the evidence. It is natural, therefore, to inquire about the positive findings which support it. Even those who regard this hypothesis with favor would agree, we believe, that supporting evidence is quite scanty.

There are a number of characteristics in which cigarette smokers are known to differ from nonsmokers and presumably more will be discovered. Thus, cigarette smokers consume more alcohol, more black coffee, change jobs more often, engage more in athletics, and are more likely to have had

at least one parent with hypertension or coronary artery disease (*34*, *51*, *56*). Discontinued cigarette smokers are weaned at a later age than those continuing to smoke (*56*). Recently, Fisher (*27*) reported that 51 monozygotic twins resembled each other more in their smoking habits than 33 dizygotic twins, thus suggesting a genetic determinant.

Two somewhat obvious, but necessary, comments on results of this type are in order: 1) The demonstration that a characteristic is related to smoking status does not by itself create a presumption that it is a common cause. It must also be shown to be related to the development of lung cancer among subgroups of individuals with the same smoking status. Alcohol and coffee fail to meet this test, while none of the other characteristics related to smoking status have been investigated from this point of view. 2) There is a quantitative question. Cigarette smokers have a ninefold greater risk of developing lung cancer than nonsmokers, while over-two-pack-a-day smokers have at least a 60-fold greater risk. Any characteristic proposed as a measure of the postulated cause common to both smoking status and lung-cancer risk must therefore be at least ninefold more prevalent among cigarette smokers than among nonsmokers and at least 60-fold more prevalent among two-pack-a-day smokers. No such characteristic has yet been produced despite diligent search.

These comments on the quantitative aspects of association apply also to the relationship of certain characteristics with lung cancer. Thus, a possible genetic basis to lung cancer has been suggested to some by the association between gastric cancer and blood group. The difference, in risk of developing gastric cancer, between blood groups A and O, however, is 20 percent, while the only study of lung cancer and blood groups (*9*) with which we are familiar shows a difference of 27 percent (and is not quite significant at the $P=0.01$ level).[7] Such differences are suggestive for further work, but cannot be considered as casting much light on differences of magnitude, ninefold to 60-fold.

Measures of Differences

The comments in the last two paragraphs have utilized a relative measure of differences in lung-cancer risk. Since Berkson (*4*) has argued that a relative measure is inappropriate in the investigation of smoking and mortality, we now discuss the use of relative and absolute measures of differences in risk. When an agent has an apparent effect on several diseases, the ranking of the diseases by the magnitude of the effect will depend on whether an absolute or a relative measure is used. Thus in Dorn's study (*20*) of American veterans there were 187 lung-cancer deaths among cigarette smokers compared with an expectation of 20 deaths, based on the rates for nonsmokers. This yields a mortality ratio of 9.35 as a relative measure and an excess of 167 deaths as an absolute measure. For cardiovascular diseases there were 1,780 deaths among

[7] Our attention has been called to a summary of three additional studies, which report no association between ABO blood groups and lung cancer, by: Roberts, J. A. F.: Blood groups and susceptibility to disease. Brit. J. Prev. & Social Med. 11: 107–125, 1957.

cigarette smokers compared to an expectation of 1,165. This gives a relative measure of 1.53 and an absolute measure of 615 deaths. Relatively, cigarettes have a much larger effect on lung cancer than on cardiovascular disease, while the reverse is true if an absolute measure is used.

Both the absolute and the relative measures serve a purpose. The relative measure is helpful in 1) appraising the possible noncausal nature of an agent having an apparent effect; 2) appraising the importance of an agent with respect to other possible agents inducing the same effect; and 3) properly reflecting the effects of disease misclassification or further refinement of classification. The absolute measure would be important in appraising the public health significance of an effect known to be causal.

The first justification for use of the relative measure can be stated more precisely, as follows:

> If an agent, A, with no causal effect upon the risk of a disease, nevertheless, because of a positive correlation with some other causal agent, B, shows an apparent risk, r, for those exposed to A, relative to those not so exposed, then the prevalence of B, among those exposed to A, relative to the prevalence among those not so exposed, must be greater than r.

Thus, if cigarette smokers have 9 times the risk of nonsmokers for developing lung cancer, and this is not because cigarette smoke is a causal agent, but only because cigarette smokers produce hormone X, then the proportion of hormone-X-producers among cigarette smokers must be at least 9 times greater than that of nonsmokers. If the relative prevalence of hormone-X-producers is considerably less than ninefold, then hormone X cannot account for the magnitude of the apparent effect (Appendix A).

The second reason for using a relative measure may be phrased as follows:

> If two uncorrelated agents, A and B, each increase the risk of a disease, and if the risk of the disease in the absence of either agent is small (in a sense to be defined), then the apparent relative risk for A, r, is less than the risk for A in the absence of B.

The presence of other real causes thus reduces the apparent relative risk. If, for example, the relative risk of developing either disease I or disease II on exposure to A is the same in the absence of other causes, and if disease I, but not disease II, also has agent B present, then the apparent relative risk of developing disease I on exposure to A will be less than that for disease II (Appendix B).

The third reason for using a relative measure is:

> If a causal agent A increases the risk for disease I and has no effect on the risk for disease II, then the relative risk of developing disease I, alone, is greater than the relative risk of developing disease I and II combined, while the absolute measure is unaffected.

Thus, in the Hammond-Horn study, the association of cigarette smoking and lung cancer was higher when only patients with a well-substantiated diagnosis of lung cancer were considered, and was lower when the group included questionable diagnoses. Using the relative risk reveals the stronger association of cigarette smoking for epidermoid-undifferentiated

carcinoma than for adenocarcinoma. The absolute measure would not differentiate between the risk for these subgroups.

The Causal Hypothesis

We turn now to a consideration of some of the contradictions in the causal hypothesis, alleged by various authors. Fisher (*25*) has stated:

> When the sexes are compared it is found that lung cancer has been increasing more rapidly in men relatively to women. . . . But it is notorious, and conspicuous in the memory of the most of us, that over the last 50 years the increase of smoking among women has been great, and that among men (even if positive) certainly small. The theory that increasing smoking is 'the cause' of the change in apparent incidence of lung cancer is not even tenable in the face of this contrast.

The available statistics do not confirm Fisher's statement. According to the Tobacco Manufacturer's Standing Committee (*76*) male per capita consumption of cigarette tobacco in Great Britain increased from 1.9 pounds in 1906 to 8 pounds in 1956. Female per capita consumption increased from essentially zero, in 1906, to 3.1 pounds in 1956. Far from making the causal hypothesis untenable, these results are entirely consistent with it, and constitute, in fact, one of the links in the chain of evidence implicating cigarettes.

The fact that cigarette smoking was associated with a higher mortality not only from lung cancer but from many other causes of death was originally considered as a contradiction by Arkin (*1*). Commenting on the first Hammond-Horn report, he wrote:

> It would thus appear that cigarette smoking is one of the causes of all ills and contributes to the over-all death rate, remembering that this rate includes such causes as accident, homicide, etc. It seems quite clear that cigarette smoking is a symptom, not a cause. It is possible—even though this is a conjecture—that the type of person who is careful of his health is less likely to be a cigarette smoker and that the cigarette smoker is likely to be the person who generally takes greater health risks.

Both the later Hammond-Horn (*38*) report and the study of American veterans (*20*) show no difference between cigarette and noncigarette smokers in mortality from accidents, violence, and suicide. If nonsmokers are biologically self-protective, it is only with respect to nonaccidental causes of death.

Berkson (*4*) also has pointed to the multiple findings in both the Hammond-Horn and the Doll-Hill results and concluded that the observed associations may have some other explanation than a causal one. He suggests three: 1) "The observed associations are 'spurious'. . . . 2) The observed associations have a constitutional basis. Persons who are nonsmokers, or relatively light smokers, are the kind of people who are biologically self-protective, and biologically this is correlated with robustness in meeting mortal stress from disease generally. 3) Smoking increases the 'rate of living' (Pearl), and smokers at a given age are, biologically, at an age older than their chronologic age."

One might ask why the finding of an association with a number of diseases, rather than just one, is necessarily contradictory and must be regarded as supporting the constitutional hypothesis. Arkin (*1*) supplied no answer, while the relevant statements of Berkson (*4*) on this point were:

> For myself, I find it quite incredible that smoking should cause all these diseases.
>
> When an investigation set up to test the theory, suggested by evidence previously obtained, that smoking causes lung cancer, turns out to indicate that smoking causes or provokes a whole gamut of diseases, inevitably it raises the suspicion that something is amiss.
>
> It is not logical to take such a set of results [*e.g.*, an association of smoking with a 'wide variety of diseases'] as confirming the theory that tobacco smoke contains carcinogenic substances which, by contact with the pulmonary tissues, initiate cancerous changes at the site of contact.

We see nothing inherently contradictory nor inconsistent in the suggestion that one agent can be responsible for more than one disease, nor are we lacking in precedents. The Great Fog of London in 1952 increased the death rate for a number of causes, particularly respiratory and coronary disease, but no one has given this as a reason for doubting the causal role of the Fog. Tobacco smoke, too, is a complex substance and consists of many different combustion products. It would be more "incredible" to find that these hundreds of chemical products all had the same effect than to find the contrary. A universe in which cause and effect always have a one-to-one correspondence with each other would be easier to understand, but it obviously is not the kind we inhabit.

The apparent multiple effects of tobacco do raise a question with respect to the mode of action, however, and since this question is related to another alleged contradiction—the apparent lack of an inhalation effect—we shall discuss them together. What mode of action, it has been asked, can one postulate to explain these diverse effects? Two remarks are in order: 1) The evidence that tobacco is a causal agent in the development of other diseases seems weaker than the evidence for lung cancer simply because the effects are smaller. While we would not exclude the possibility that cigarettes play a causal role in, for instance, the development of arteriosclerotic-coronary heart disease, the possibility that a common third factor will be discovered, which explains a 70 percent elevation in risk from coronary heart disease among cigarette smokers, is less remote than the possibility that the ninefold risk for lung cancer will be so explained. 2) Accepting, for the sake of discussion, the causal role of cigarettes for any disease showing an elevated mortality ratio, no matter how small, the presence of other causes will be manifested in a lowered mortality ratio. Thus, even if cigarette consumption causes an elevation of 70 percent in mortality from coronary heart disease, other causes of great importance must also be present, as is manifested by the high mortality from this disease among nonsmokers. The existence of a small number of nonsmokers who develop lung cancer is a definite indication, by the same token, that cigarettes are not an absolutely necessary condition and that there are other causes of lung cancer.

If tobacco smoke does have multiple effects, each of these effects must be studied separately because of the complex nature of the agent. To postulate in advance that a single mode of action will be found to characterize them all is an unwarranted oversimplification. It is generally accepted, for example, that tobacco smoke causes thromboangiitis obliterans in susceptible humans by interfering with the peripheral circulation, and that it causes tumors when painted on the backs of susceptible mice because of the presence of carcinogens in the tars. The *a priori* postulation of a single mode of action for these two effects is no substitute for detailed study of each.

As to the possible mode of action of tobacco smoke in inducing lung cancer, the evidence at this writing suggests direct action of substances in tobacco smoke on susceptible tissues with which they are in contact. Aside from background knowledge derived from experimental carcinogenesis which suggests this explanation, the following evidence favors it: 1) Cigarette smoke, which is usually drawn into the lungs, is associated with mortality from lung cancer, while smoke from pipes and cigars, which is usually not inhaled, is not. 2) For sites with which smoke is in direct contact, whether or not inhaled, particularly buccal cavity and larynx, the type of tobacco used makes less difference in incidence. 3) In experimental carcinogenesis, which uses tobacco tars, tumors have appeared at the site of application, and their incidence has not been seriously dependent on the type of tobacco used. 4) The relative risk of lung cancer is higher among cigarette smokers who inhale than among those smoking the same number of cigarettes per day, but who do not inhale.

Several critics (*26*, *43*, *54*) have stressed the failure of Doll and Hill (*17*), in their preliminary report, to find a difference in risk between inhalers and noninhalers, but this finding was contradicted in three other studies (*8*, *50*, *68*). Further work on this point is desirable, but would be more convincing if a more objective measure were found of the amount of smoke to which human bronchial epithelium is exposed in the course of smoking a cigarette.

Why, it is sometimes asked, do most heavy cigarette smokers fail to develop lung cancer if cigarettes are in fact a causal agent? We have no answer to this question. But neither can we say why most of the Lübeck babies who were exposed to massive doses of virulent tubercle bacilli failed to develop tuberculosis. This is not a reason, however, for doubting the causal role of the bacilli in the development of the disease.

One cannot discuss the mode of action of tobacco without becoming aware of the necessity of vastly expanded research in the field. The idea that the subject of tobacco and mortality is a closed one requiring no further study is not one we share. As in other fields of science, new findings lead to new questions, and new experimental techniques will continue to cast further light on old ones. This does not imply that judgment must be suspended until all the evidence is in, or that there are hierarchies of evidence, only some types of which are acceptable. The doctrine that

one must never assess what has already been learned until the last possible piece of evidence would be a novel one for science.

It would be desirable to have a set of findings on the subject of smoking and lung cancer so clear-cut and unequivocal that they were self-interpreting. The findings now available on tobacco, as in most other fields of science, particularly biologic science, do not meet this ideal. Nevertheless, if the findings had been made on a new agent, to which hundreds of millions of adults were not already addicted, and on one which did not support a large industry, skilled in the arts of mass persuasion, the evidence for the hazardous nature of the agent would be generally regarded as beyond dispute. In the light of all the evidence on tobacco, and after careful consideration of all the criticisms of this evidence that have been made, we find ourselves unable to agree with the proposition that cigarette smoking is a harmless habit with no important effects on health or longevity. The concern shown by medical and public health authorities with the increasing diffusion to ever younger groups of an agent that is a health hazard seems to us to be well founded.

Appendix A

We feel obliged to give proof of the rather obvious statement on the magnitudes of relative risk because it has been suggested that the use of a relative measurement is merely "instinctive" and lacking in rational justification. Let the disease rate for those exposed to the causal agent, B, be r_1 and for those not exposed, r_2, each rate being unaffected by exposure or nonexposure to the noncausal agent, A. Let $r_1 > r_2$. Of those exposed to A, let the proportion exposed to B be p_1, and of those not exposed to A, let the proportion exposed to B be p_2. Because of the assumed positive correlation between A and B, $p_1 > p_2$. Then

R_1 = rate for those exposed to A $= p_1 r_1 + (1 - p_1) r_2$

R_2 = rate for those not exposed to A $= p_2 r_1 + (1 - p_2) r_2$

$$(1)\quad \frac{R_1}{R_2} = \frac{p_1 r_1 + (1 - p_1) r_2}{p_2 r_1 + (1 - p_2)\, r_2}.$$

Since $p_1 > p_2$ and $r_1 > r_2$, it follows that $R_1/R_2 > 1$.

From (1) we obtain

$$\frac{p_1}{p_2} = \frac{R_1}{R_2} + \frac{1}{p_2}\ \frac{r_2}{r_1}\quad \left[(1 - p_2)\frac{R_1}{R_2} - (1 - p_1)\right].$$

Since $p_1 > p_2$ and $R_1/R_2 > 1$, the second term on the right is positive and

$$\frac{p_1}{p_2} > \frac{R_1}{R_2}.$$

Since p_1/p_2 is the ratio of the prevalence of B among those exposed to A relative to that among those not so exposed, and R_1/R_2 is the apparent relative risk, r, the statement is proved.

On the other hand, if the absolute difference, $R_1 - R_2$, is used, the relationship,

$$(R_1 - R_2) = (r_1 - r_2)\ (p_1 - p_2)$$

leads to no useful conclusion about $p_1 - p_2$.

Appendix B

The proof again is simple. Let r_{11} denote the risk of the disease in the presence of both A and B, r_{12}, the risk in the presence of A and absence of B, r_{21}, the risk in the absence of A and presence of B, and r_{22} the risk in the absence of both A and B. It is reasonable to assume $r_{22} = 0$, but the less restrictive specification $r_{22} < r_{12}r_{21}/r_{11}$ is sufficient for what follows. The proportion of the population exposed to B is denoted by p, and this, by hypothesis, is the same whether A is present or absent. Then

R_1 = rate for those exposed to A $= pr_{11} + (1 - p)r_{12}$

R_2 = rate for those not exposed to A $= pr_{21} + (1 - p)r_{22}$

and

$$\frac{R_1}{R_2} = \text{apparent relative risk} = \frac{r_{12}}{r_{22}} \frac{\frac{p}{1-p}\frac{r_{11}}{r_{12}} + 1}{\frac{p}{1-p}\frac{r_{21}}{r_{22}} + 1}.$$

Since $r_{22}/r_{21} < r_{12}/r_{11}$, the second factor is less than unity and

$$\frac{R_1}{R_2} < \frac{r_{12}}{r_{22}},$$

which proves the proposition.

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Announcements

The Japan Cancer Society

The most common cause of death among the Japanese is cerebral hemorrhage, and second is cancer. In 1957, 83,029 Japanese died of cancer: the death rate was 91.2 per 100,000—twice that of 1900. Cancer is on the increase at a time when other major diseases are either remaining at the same level or declining. Cancer of the stomach accounts for 54 percent of cancer deaths among men and 39 percent among women.

For many years the Japanese have stressed the importance of establishing a nongovernmental body to lead the campaign against cancer, since the only private organization was the Japanese Foundation for Cancer Research. In October 1957, at a general meeting of the Japanese Cancer Association, an appeal was made by Doctor Hiroshige Shiota for such an organization. After this meeting, the *Asahi Shimbun-sha* (a leading newspaper chain), the Japanese Medical Association, the Welfare Ministry, and other prominent individuals promised their support and aid, with the result that on August 1, 1958, the Japan Cancer Society was incorporated.

The Society has 15 directors and 3 auditors chosen from among leading scholars and scientists in the field of cancer research and leaders in other fields. Hiroshige Shiota (Professor Emeritus, Tokyo University) is Chairman of the Board of Directors; Keizo Shibusawa (former Finance Director, Advisor to the Foreign Ministry) Managing Director; Shintaro Ryu (Executive Director, *Asahi Shimbun-sha*) Executive Director.

The aims of the Society include surveys and research on the etiology, prevention, and treatment of cancer; the establishment of cancer institutions and centers; training of doctors and other personnel; publication of results of research; public information on cancer through all available media; and liaison and cooperation with foreign organizations concerned with cancer.

The Society's plans for the first year particularly emphasize research and surveys on the mechanism of cancer cell formation, epidemiologic studies, treatment of cancer with radioactive isotopes, and effective chemotherapy against cancer.

The Society is located in the *Asahi Shimbun-sha* newspaper offices, 3, 2-chome, Yuraku-cho, Chiyoda-ku, Tokyo. Local branches will be set up wherever necessary; six have already been suggested.

Symposium on the Biochemical Cytology of Liver

As part of its 2-day meeting in Atlantic City, New Jersey, the Histochemical Society will hold a Symposium entitled "The Biochemical Cytology of Liver," on the morning of April 13, 1959, in Convention Hall. Chaired by the Society's President, A. B. Novikoff, the program will include papers by A. Ficq on the nucleus, T. Peters, Jr., on cytoplasmic particles, and K. R. Porter on fine structure changes induced by azo-dye feeding. Short presentations will be given by L. L. Miller, R. E. Shank, A. B. Novikoff, M. Simpson, K. Paigen, I. M. Arias, and E. H. Leduc. Open discussion is planned. Details will be found in the January 1959 issue of the *Journal of Histochemistry and Cytochemistry*.

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